(54) Title: ENHANCEMENT OF VASCULAR FUNCTION BY MODULATION OF ENDOGENOUS NITRIC OXIDE PRODUCTION OR ACTIVITY
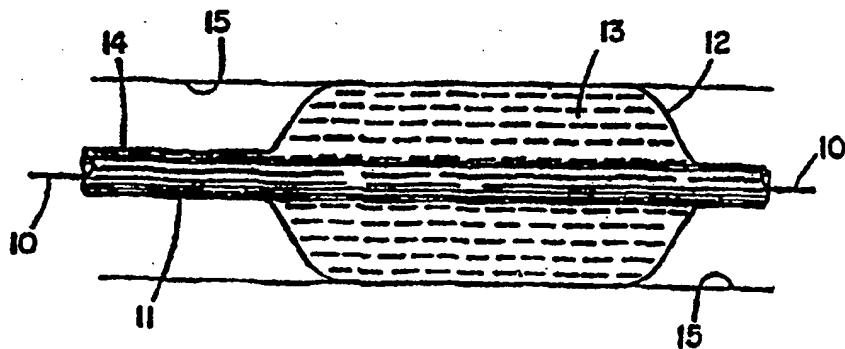
(57) Abstract

Vascular function and structure is maintained or improved by long term administration of physiologically acceptable compounds which enhance the level of endogenous nitric oxide or other intermediates in the NO induced relaxation pathway in the host. Alternatively, or in combination, other compounds may be administered which provide for short term enhancement of nitric oxide, either directly or by physiological processes. An intramural process is provided for preventing restenosis by introducing precursor amino acid(s) through the balloon lumen (14) into the interior chamber (13) of the balloon (12). During inflation, the balloon (12) presses outwardly against the vessel wall (15) and the precursor amino acid(s) are introduced into the vessel wall (15).

ENHANCEMENT OF VASCULAR FUNCTION BY MODULATION OF ENDOGENOUS NITRIC OXIDE PRODUCTION OR ACTIVITY

INTRODUCTION

This invention was supported in part by the United States Government under Grant 1K07HC02660 (NHLBI). The
5 U.S. Government may have an interest in this application.

Technical Field

The field of this invention is the modulation of NO activity, which finds application in maintaining and
10 improving vascular function and thereby preventing or improving vascular degenerative diseases.

Background

Atherosclerosis and vascular thrombosis are a major
15 cause of morbidity and mortality, leading to coronary artery disease, myocardial infarction, and stroke. Atherosclerosis begins with an alteration in the endothelium, which lines the blood vessels. The endothelial alteration results in adherence of monocytes,
20 which penetrate the endothelial lining and take up residence in the subintimal space between the endothelium and the vascular smooth muscle of the blood vessels. The monocytes absorb increasing amounts of cholesterol (largely in the form of oxidized or modified low-density
25 lipoprotein) to form foam cells. Oxidized low-density lipoprotein (LDL) cholesterol alters the endothelium, and the underlying foam cells distort and eventually may even rupture through the endothelium.

Platelets adhere to the area of endothelial
disruption and release a number of growth factors,
including platelet derived growth factor (PDGF). PDGF,
which is also released by foam cells and altered
5 endothelial cells, stimulates migration and proliferation
of vascular smooth muscle cells into the lesion. These
smooth muscle cells release extracellular matrix (collagen
and elastin) and the lesion continues to expand.
Macrophages in the lesion elaborate proteases, and the
10 resulting cell damage creates a necrotic core filled with
cellular debris and lipid. The lesion is then referred to
as a "complex lesion." Rupture of this lesion can lead to
thrombosis and occlusion of the blood vessel. In the case
of a coronary artery, rupture of a complex lesion may
15 precipitate a myocardial infarction, whereas in the case
of a carotid artery, stroke may ensue.

One of the treatments that cardiologists and other
interventionalists employ to reopen a blood vessel which
is narrowed by plaque is balloon angioplasty
20 (approximately 300,000 coronary and 100,000 peripheral
angioplasties are performed annually). Although balloon
angioplasty is successful in a high percentage of the
cases in opening the vessel, it unfortunately denudes the
endothelium and injures the vessel in the process. This
25 damage causes the migration and proliferation of vascular
smooth muscle cells of the blood vessel into the area of
injury to form a lesion, known as myointimal hyperplasia
or restenosis. This new lesion leads to a recurrence of
symptoms within three to six months after the angioplasty
30 in a significant proportion of patients (30-40%).

In atherosclerosis, thrombosis and restenosis there
is also a loss of normal vascular function, such that
vessels tend to constrict, rather than dilate. The
excessive vasoconstriction of the vessel causes further
35 narrowing of the vessel lumen, limiting blood flow. This
can cause symptoms such as angina (if a heart artery is

2

involved), or transient cerebral ischemia (i.e. a "small stroke", if a brain vessel is involved). This abnormal vascular function (excessive vasoconstriction or inadequate vasodilation) occurs in other disease states as

5   well. Hypertension (high blood pressure) is caused by excessive vasoconstriction, as well as thickening, of the vessel wall, particularly in the smaller vessels of the circulation. This process may affect the lung vessels as well causing pulmonary (lung) hypertension. Other

10   disorders known to be associated with excessive vasoconstriction, or inadequate vasodilation include transplant atherosclerosis, congestive heart failure, toxemia of pregnancy, Raynaud's phenomenon, Prinzmetal's angina (coronary vasospasm), cerebral vasospasm,

15   hemolytic-uremia and impotence.

Because of their great prevalence and serious consequences, it is critically important to find therapies which can diminish the incidence of atherosclerosis, vascular thrombosis, restenosis, and these other disorders

20   characterized by abnormality of vascular function and structure. Ideally, such therapies would inhibit the pathological vascular processes associated with these disorders, thereby providing prophylaxis, retarding the progression of the degenerative process, and restoring

25   normal vasodilation.

As briefly summarized above, these pathological processes are extremely complex, involving a variety of different cells which undergo changes in their character, composition, and activity, as well as in the nature of the

30   factors which they secrete and the receptors that are up- or down-regulated. A substance released by the endothelium, "endothelium derived relaxing factor" (EDRF), may play an important role in inhibiting these pathologic processes. EDRF is now known to be nitric oxide (NO) or a

35   labile nitroso compound which liberates NO. (For purposes of the subject invention, unless otherwise indicated,

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nitric oxide (NO) shall intend nitric oxide or the labile nitroso compound which liberates NO.) This substance relaxes vascular smooth muscle, inhibits platelet aggregation, inhibits mitogenesis and proliferation of
5 cultured vascular smooth muscle, and leukocyte adherence. Because NO is the most potent endogenous vasodilator, and because it is largely responsible for exercise-induced vasodilation in the conduit arteries, enhancement of NO synthesis could also improve exercise capacity in normal
10 individuals and those with vascular disease. NO may have other effects, either direct or indirect, on the various cells associated with vascular walls and degenerative diseases of the vessel.

15 Relevant Literature

Girerd et al. (1990) *Circulation Research* 67:1301-1308 report that intravenous administration of L-arginine potentiates endothelium-dependent relaxation in the hind limb of cholesterol-fed rabbits. The authors conclude
20 that synthesis of EDRF can be increased by L-arginine in hypercholesterolemia. Rossitch et al. (1991) *J. Clin. Invst.* 87:1295-1299 report that *in vitro* administration of L-arginine to basilar arteries of hypercholesterolemic rabbits reverses the impairment of endothelium-dependent
25 vasodilation and reduces vasoconstriction. They conclude that the abnormal vascular responses in hypercholesterolemic animals is due to a reversible reduction in intracellular arginine availability for metabolism to nitric oxide.
30 Creager et al. (1992) *J. Clin. Invest.* 90:1248-1253, report that intravenous administration of L-arginine improves endothelium-derived NO-dependent vasodilation in hypercholesterolemic patients.

Cooke et al., "Endothelial Dysfunction in
35 Hypercholesterolemia is Corrected by L-arginine," Endothelial Mechanisms of Vasomotor Control, eds.

4

Drexler, Zeiher, Bassenge, and Just; Steinkopff Verlag
Darmstadt, 1991, pp. 173-181, review the results of the
earlier references and suggest, "If the result of these
investigations may be extrapolated, exogenous
5 administration of L-arginine (i.e., in the form of dietary
supplements) might represent a therapeutic adjunct in the
treatment and/or prevention of atherosclerosis".

Cooke (1990) *Current Opinion in Cardiology* 5:637-644
discusses the role of the endothelium in the
10 atherosclerosis and restenosis, and the effect that these
disorders have on endothelial function.

Cooke (1992) *J. Clin. Invest.* 90:1168-1172, describe
the effect of chronic administration of oral L-arginine in
hypercholesterolemic animals on atherosclerosis. This is
15 the first demonstration that oral L-arginine supplements
can improve the release of NO from the vessel wall. The
increase in NO release from the vessel wall was associated
with a striking reduction in atherosclerosis in
hypercholesterolemic animals. This is the first evidence
20 to support the hypothesis that increasing NO production by
the vessel wall inhibits the development of
atherosclerosis.

Cooke and Tsao (1992) *Current Opinion in Cardiology*
7:799-804 describe the mechanism of the progression of
25 atherosclerosis and suggest that inhibition of nitric
oxide may disturb vascular homeostasis and contribute to
atherogenesis.

Cooke and Santosa (1993) In: Steroid Hormones and
Dysfunctional Bleeding, AAAS Press, review the activities
30 of EDRF in a variety of roles and suggest that
reversibility of endothelial dysfunction may be affected
by the stage of atherosclerosis. They conclude that EDRF
is a potent vasodilator, plays a key role in modulating
conduit and resistance vessel tone, has important effects
35 on cell growth and interactions of circulatory blood cells
with a vessel wall, and that disturbances of EDRF activity

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may initiate or contribute to septic shock, hypertension, vasospasm, toxemia and atherosclerosis.

Fitzpatrick et al., *American Journal of Physiology* 265 (Heart Circ. Physiol. 34):H774-H778, 1993 report that
5 wine and other grape products may have endothelium-dependent vasorelaxing activity *in vitro*.

Wang et al. (1994) *J. Am. Cell. Cardiol.* 23:452-458, report that oral administration of arginine prevents atherosclerosis in the coronary arteries of
10 hypercholesterolemic rabbits.

Drexler et al. (1994) *Circulation* 89:1615-1623 describe the effect of intravenous arginine upon coronary vascular tone. This was the first evidence that intravenous arginine could restore normal NO-dependent
15 vasodilation in the coronary arteries of patients with cardiac transplants, Tsao et al. (1994) *Circulation* 89:2176-2182 demonstrates that oral administration of arginine to hypercholesterolemic rabbits enhances the release of nitric oxide by the vessel wall, and inhibits
20 monocytes from sticking to the vessel.

Tsao et al. (1994) *J. Arterioscl. Thromb.* 14:1529-1533 reveals that oral arginine administration to hypercholesterolemic rabbits inhibits platelet aggregation (blood clotting). Platelet aggregation plays an important
25 role in causing vascular thrombosis in vascular degenerative disorders.

Von de Leyen et al. (1995) *PNAS USA*, show that the gene encoding nitric oxide synthase (the enzyme that produces NO) can be inserted into the carotid artery of
30 the rat. This causes the rat carotid artery to make more NO, and thereby enhances vasodilation and inhibits thickening of the vessel wall after balloon angioplasty.

Noruse et al. (1994) *Arterioscler. Thromb.* 14:746-752, report that oral administration of an antagonist of
35 NO production accelerates atherogenesis in hypercholesterolemic rabbits.

6

Cayette et al. (1994) *Arterioscler. Thromb.* **14**:753-759, also report that oral administration of an antagonist of NO production accelerates plaque development in hypercholesterolemic rabbits.

5   Other references which refer to activities attributed to NO or its precursor include: Pohl and Busse (1989) *Circ. Res.* **65**:1798-1803; Radomski et al. (1987) *Br. J. Pharmacol.* **92**:181-187; Stamler et al. (1989) *Circ. Res.* **65**:789-795; anti-platelet activity); Garg and Hassid

10   (1989) *J. Clin. Invest.* **83**:1774-1777; Weidinger et al. (1990) *Circulation* **81**:1667-1679; NO activity in relation to vascular smooth muscle growth); Ross (1986) *N. Engl. J. Med.* **314**:488-500; Bath et al. (1991) *Arterioscler. Thromb.* **11**:254-260; Kubes et al. (1991) *Proc. Natl. Acad. Sci. USA*

15   **89**:6348-6352; Lefer et al. (1990) In: Endothelium-Derived Contracting Factors. Basel, S. Karger, pp.190-197; NO activity in relation to leukocyte adhesion and migration); Heistad et al. (1984) *Circ. Res.* **43**:711-718; Rossitch et al. (1991) *J. Clin. Invest.* **87**:1295-1299; Yamamoto et al.

20   (1988) <u>ibid</u> **81**:1752-1758; Andrews et al. (1987) *Nature* **327**:237-239; Tomita et al. (1990) *Circ. Res.* **66**:18-27; Kugiyama et al. (1990) *Nature* **344**:160-162; Mitchell et al. (1992) *J. Vasc. Res.* **29**:169 (abst.); Minor et al. (1990) *J. Clin. Invest.* **86**:2109-2116; NO activity in relation to

25   hypercholesterolemia); Tanner et al. (1991) *Circulation* **83**:2012-2020; Kuo et al. (1992) *Circ. Res.* **70**:f465-476; Drexler et al. (1991) *Lancet* **338**:1546-1550; Schuschke et al. (1994) *Int. J. of Microcircu: Clin. and Exper.* **14**(4):204-211; Yao et al. (1992) *Circulation* **86**:1302-1309;

30   Higashi et al. (1995) *Hypertension* **25**(4 Pt 2):898-902; Kharitonov et al. (1995) *Clin. Sci.* **88**(2):135-139; Smulders et al. (1994) *Clin. Sci.* **87**(1):37-43; Bode-boger et al. (1994) *Clin. Sci.* **87**(3):303-310; Bode-Boger et al. (1994) *Clin. Sci.*; Randall et al. (1994) *Clin. Sci.*

35   **87**(1):53-59; Dubois-Rande et al. (1992) *J. Card. Pharm.* **20** Suppl. **12**:S211-3; Otsuji et al. (1995) *Am. Heart J.*

7

129(6): 1094-1100; Nakanishi et al. (1992) *Am. J. of*
Physio. 263(6 Pt 2):H1650-8; Kuo et al. (1992) *Circ.*
Research 70(3): 465-476; Tanner et al. (1991) *Circulation*
83(6):2012-2020; Meng et al. (1995) *J. Am. Col. Card.*
5   25(1):269-275; Lefer and Ma (1993) *Arterioscl. and Thromb.*
13(6):771-776; McNamara et al. (1993) *Biochem. and*
Biophys. Res. Comm. 193(1):291-296; Tarry and Makhoul
(1994) *Arter. and Thromb.* 14(6):983-943; Davies et al.
(1994) *Surgery* 116(3):557-568; and Raij (1994) *Kidney*
10 *Institute* 45:775-781.

SUMMARY OF THE INVENTION

Methods are provided for improving vascular function
and structure, particularly modulating vascular
15 relaxation, cellular adhesion, infiltration and
proliferation by modulating the level of nitric oxide or
active precursor at a physiological site. The methods
find use in preventing the degradation of vascular
function, particularly as involved with the occurrence of
20 atherosclerosis, restenosis, thrombosis, hypertension,
impotence, and other disorders characterized by reduced or
inadequate vasodilation. The enhancement of endogenous
nitric oxide or secondary messenger availability at a
physiological site improves vascular relaxation and
25 thereby relieves symptoms due to inadequate blood flow
(such as angina) and can counteract inappropriate
elevation of blood pressure. The enhancement of
endogenous nitric oxide also inhibits initiation and the
progression of atherosclerosis, restenosis, vascular
30 hypertrophy or hyperplasia and thrombosis. This is due to
the fact that nitric oxide is not only a potent modulator,
but can also inhibit platelets and white blood cells from
adhering to the vessel wall. As a prophylaxis or
treatment for vascular function deterioration,
35 particularly in atherosclerotic susceptible hosts, the
agent is chronically administered at an effective dosage.

8

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For restenosis, the agent may be administered for a limited period since this pathological process generally abates 3-6 months after the vascular injury (i.e. angioplasty or atherectomy). Oral administration of

5 L-lysine, precursors to L-lysine, e.g. oligopeptides or polypeptides comprising L-lysine, or proteins comprising high levels of L-lysine, by itself or in combination with L-arginine, particularly further supplemented with GRAS substances which enhance the effectiveness of the active

10 agents, as a dietary supplement, will increase NO elaboration and thereby diminish the effects of atherogenesis. Other techniques to enhance NO or secondary messenger availability may be utilized such as increasing the availability of NO synthase, for example,

15 as a result of enhanced expression of NO synthase in the vessel wall, particularly at the lesion site, release of NO from the vessel wall or reduction of degradation of NO or the secondary messenger, cyclic guanosine monophosphate ("cGMP").

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar diagram of histomorphometric studies of the effect of L-arginine on atherosclerotic plaque in hypercholesterolemic animals. (See Ex. 1)

25   Figure 2 is a nephelometric scan of the effect of L-arginine diet supplement on platelet reactivity as evidenced by platelet aggregation initiated by adenosine diphosphate. (See Ex. 2) A) aggregation of platelets from hypercholesterolemic rabbit; B) reduced aggregation of

30 platelets from hypercholesterolemic rabbit treated with L-arginine; C) antagonism of NO synthase by LNMMA reverses the beneficial effect of L-arginine.

Figure 3 is a bar diagram comparing the effect of L-arginine diet supplement on cell binding to aortic

35 endothelium of hypercholesterolemic animals. (See Ex. 4)

9

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Figure 4. Lesion surface area of thoracic aortae
from all arginine treated hypercholesterolemic animals
(ARG, weeks 14-23) is reduced in comparison to that of
hypercholesterolemic animals receiving vehicle (CHOL,
5   weeks 14-23). (See Ex. 5)

Figure 5. Macrophage accumulation in iliac arteries
4 weeks following balloon injury. (Macrophage
infiltration into the vessel wall initiates and
accelerates plaque formation). Data is expressed as a
10   percent of the vessel that contain macrophages. Balloon
injury in hypercholesterolemic rabbits (CHOL) results in a
marked increase in arterial macrophage accumulation
compared with injured iliac arteries from rabbits on
normal chow (CONT). Macrophage accumulation in iliac
15   arteries from hypercholesterolemic rabbits receiving L-
arginine (ARG) is significantly reduced compared to the
CHOL group. (*; $p<0.01$, ARG v. CHOL). This study
revealed that oral arginine treatment markedly reduced the
infiltration of monocytes/macrophages into the vessel
20   wall, explaining in part the effect of arginine to inhibit
plaque formation. (See Ex. 6)

Figure 6. Stimulation of cultured endothelial cells
with fluid flow causes them to secrete nitric oxide.
Flow-induced secretion of nitric oxide decreases
25   endothelial adhesiveness induced by oxidized LDL
cholesterol (oxLDL; 30 µg/ml). Exposure of human aortic
endothelial cells to oxLDL increased the *ex vivo* binding
of monocytes when compared to Control. In comparison to
cells not exposed to flow (static), previous exposure to
30   flow inhibited the monocyte adhesion induced by oxLDL.
These effects of flow were blocked by NO synthase
inhibitors and mimicked by NO donors (PAPA-NO) or cyclic
GMP (cGMP). Bars represent mean ± SEM. *$p<0.05$; **$p<0.01$.
(See Ex. 8)
35   Figure 7 is a bar diagram of morphometric
measurements of intimal lesion thickening two weeks after

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a balloon angioplasty in animals treated with a plasmid
construct containing the gene for NO synthase (INJ+NOS) in
comparison to control vector (INJ+CV) or untreated injured
vessels (INJ). (See Ex. 11)

5   Figure 8 is a histogram showing the effect of local
intraluminal administration of arginine on restenosis.
Hypercholesterolemic rabbits had balloon angioplasty of
the iliac artery. Immediately thereafter some animals
received an infusion of arginine directly into the vessel

10 by means of a catheter designed to apply high local
concentrations of arginine to the vessel. Two to four
weeks later, vessels were removed from the animals, and
examined microscopically. Thickening of the vessel wall
(internal thickening or "restenosis") was reduced in the

15 animals treated with intraluminal infusion of arginine
(ARG) in comparison to those treated with vehicle. (See
Ex. 12)

        Figure 9 is a set of dose-response curves showing the
effect of chronic lysine administration on endothelium

20 dependent vasodilation in hypercholesterolemic rabbits.
Chronic oral administration of lysine (for ten weeks)
improved NO-mediated vasodilation; this improvement in NO
activity was also associated with a marked reduction in
plaque area. Chronic administration of lysine was just as

25 effective as arginine in restoring vascular function and
structure. (See Ex. 14)

        Figure 10 is a scatter-diagram illustrating the
relationship between the level of blood LDL-cholesterol
and monocyte binding. Monocytes were isolated from the

30 blood of humans with normal or elevated cholesterol
levels. The binding of these monocytes to endothelial
cells in culture was observed. Monocytes from individuals
with high cholesterol levels have a greater adhesiveness
for endothelial cells. This monocyte-endothelial cell

35 interaction *in vivo* is the first step in the development
of atherosclerotic plaque. (See Ex. 15)

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Figure 11 is a bar diagram showing the adhesiveness of monocytes obtained from subjects with normal cholesterol levels (CONT) and those from hypercholesterolemic (HC) humans, before, during, and

5 after treatment with arginine (the NO precursor). Prior to initiating arginine (Arg) or placebo (plac) treatment, monocytes from hypercholesterolemic individuals have a greater tendency to bind to endothelial cells *ex vivo* (baseline). After 2 weeks of arginine treatment monocytes

10 from these hypercholesterolemic individuals have a significantly reduced adhesiveness and are no different from those of the normal subjects. At this point arginine therapy was discontinued and there was a washout (4 weeks). At this time point, monocytes from the patients

15 previously treated with arginine now have increased adhesiveness, off of the arginine treatment. (See Ex. 15)

Figure 12 is a bar diagram which shows that monocytes from individuals with elevated cholesterol (CHOL) have greater adhesiveness for endothelial cells. However,

20 after treatment with sodium nitroprusside (CHOL + SNP), the adhesiveness of these monocytes is normalized. SNP is an NO donor. (See Ex. 15)

Figure 13 is a set of histograms showing aggregation of platelets obtained in hypercholesterolemic humans (hc),

25 and individuals with normal cholesterol levels (nc). Platelet aggregation *ex vivo* in response to adenosine diphosphate (ADP) is increased in hc individuals in comparison to normal individuals. After 2 weeks of treatment with oral L-arginine, platelet aggregation is

30 attenuated in the hypercholesterolemic individuals, while an even greater effect of the treatment is seen at four weeks. (See Ex. 16)

Figure 14 is a bar graph showing increases in coronary blood flow in response to intracoronary infusions

35 of acetylcholine (ACH) before and after intravenous infusion of L-arginine (30 g), in patients with transplant

12

atherosclerosis. Acetylcholine stimulates the release of
NO from the vessel wall causing vasodilation and increased
blood flow. There is improved NO-dependent vasodilation
after L-arginine administration. (See Ex. 18)

5   Figure 15 is a fragmentary view, partially in
section, of a drug delivery apparatus for use in the
subject invention positioned in a blood vessel with the
dilatation balloon in its inflated state and containing a
solution of an NO precursor.

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DESCRIPTION OF SPECIFIC EMBODIMENTS

In accordance with the subject invention, vascular
function is maintained or its deterioration inhibited or
retarded by enhancing the level or activity of endogenous
15 nitric oxide. By enhancing the level or activity of
endogenous nitric oxide, common vascular degenerative
diseases such as atherosclerosis, restenosis,
hypertension, vasospasm, impotence, angina, and vascular
thrombosis, can be treated prophylactically and/or
20 therapeutically. The enhanced level or activity of nitric
oxide (which is intended to include any precursor of
nitric oxide which results in such enhanced level) can be
achieved by modulating the activity, synthesis or
concentration of any of the components associated with the
25 formation of nitric oxide in the nitric oxide synthetic
pathway, or inhibiting the rate of degradation of nitric
oxide, its precursors, or the secondary messengers
associated with the relaxation signal. In referring to
the enhanced level or activity, the term "effect" will be
30 used to encompass the two situations. The enhanced effect
of nitric oxide may be a result of oral or intravenous
administration to the patient of a precursor in the
metabolic pathway to the production of nitric oxide,
particularly an amino acid (such as L-lysine, by itself or
35 in combination with L-arginine {L-lysine, by itself or in
combination with L-arginine, will be referred to as the

13

"amino acid(s)" or "NO precursor amino acid(s)},
polypeptides comprising these amino acids, and the like),
providing an enzyme in the metabolic pathway to NO,
particularly NO synthase, by introduction of the gene for
5   NO synthase under conditions for integration of the gene
into the endothelial or other cells and expression of the
gene, or by directly adding an enzyme associated with the
production of nitric oxide. The enhanced level of nitric
oxide may also result from administration of an agent to
10 protect the NO from degradation, such as an oxidant,
reductant or superoxide dismutase. Alternatively, the
agent may serve to enhance the bioavailability or
effectiveness of the primary active agent, such as L-
lysine or L-arginine. The agent, individually or in
15 combination, will be administered in a form of other than
a natural food source, such as meat or plants as natural
protein sources, fruits or products derived therefrom.
When the amino acid(s) is the agent, the agent is supplied
as a dietary supplement to enhance the normal daily dosage
20 of the amino acid and not as the primary source of the
amino acid. Usually, the enhancement will be at least
25%, more usually at least 50% and may be 100% or more,
preferably at least about 75%.

One approach is to employ L-lysine, as an individual
25 amino acid, or in combination with L-arginine, or as a
precursor to L-lysine, e. g. a monomer or a polypeptide,
as a dietary supplement. The amino acid(s) are
administered as any physiologically acceptable salt, such
as the hydrochloride salt, glutamate salt, etc. They can
30 also be administered as a peptide (e.g., poly-L-lysine,
poly-L-arginine, or combinations thereof) so as to
increase plasma levels of the NO precursor. Oligopeptides
of particular interest include oligopeptides of from 2 to
30, usually 2 to 20, preferably 2 to 10 amino acids,
35 having at least 10 mol % of L-lysine, usually at least
about 25 mol %, more usually at least about 50 mol % of L-

14

lysine, generally having a total of at least about 50 mol
% of L-lysine and L-arginine, preferably at least about
75 mol % of L-lysine and L-arginine, more preferably
having at least about 95 mol % of L-lysine and L-arginine.
5   The oligopeptides can be modified by being ligated to
other compounds, which can enhance absorption from the
gut, provide for enhancement of NO synthesis or stability,
e.g. reducing agents and antioxidants, and the like.
        Naturally occurring sources include protamine or
10 other naturally occurring NO precursor amino acid(s)
containing protein, which is high in L-lysine or both of
the indicated amino acids, e.g. greater than about 40%,
preferably greater than about 50%.   The amount of the
L-lysine present will provide at least 25%, more usually
15 at least about 50%, of the therapeutic dosage desired for
affecting the pathways associated with atherosclerosis and
restenosis. As indicated, L-lysine may provide 100% of
the therapeutic dosage, but desirably a combination of L-
lysine and L-arginine will be used.
20   The administration of L-lysine, other convenient NO
precursor, or other agent which enhances NO availability,
would be in accordance with a predetermined regimen, which
would be at least once weekly and over an extended period
of time, generally at least one month, more usually at
25 least three months, and as a chronic treatment, could last
for one year or more, including the life of the host. The
dosage administered will depend upon the frequency of the
administration, the blood level desired, other concurrent
therapeutic treatments, the severity of the condition,
30 whether the treatment is for prophylaxis or therapy, the
age of the patient, the natural level of NO in the
patient, and the like. Desirably, the amount of the NO
precursor amino acid(s) (K and R) or biologically
equivalent compound which is used would generally provide
35 a plasma level in the range of about 0.15 to 30mM. The
oral administration of K and R can be achieved by

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providing K and R, other NO precursor, or NO enhancing
agent as a pill, powder, capsule, liquid solution or
dispersion, particularly aqueous, or the like. Various
carriers and excipients may find use in formulating the NO
5 precursor, such as lactose, terra alba, sucrose, gelatin,
aqueous media, physiologically acceptable oils, e.g.
peanut oil, and the like. Usually, if daily, the
administration of K and R for a human host as a dietary
supplement will be about 1 to 12g per day. If used as the
10 sole source of the amino acids, the administration will be
from about 5 to 20g per day.

Furthermore, other agents can be added to the oral
formulation of the amino acids or polypeptides to enhance
their absorption, and/or to enhance the activity of NO
15 synthase, e.g. B_6 (50-250 mg/d), folate (0.4-10 mg per
daily dose), B_{12} (0.5-1 mg/d) or calcium (250-1000 mg per
daily dose). Furthermore, agents known to protect NO from
degradation, such as antioxidants (e.g. cysteine or N-
acetyl cysteine 200-1000 mg/d Vitamin C (250-2000 mg daily
20 dose), (coenzyme Q 25-90 mg daily dose, glutathione 50-250
mg daily dose), Vitamin E (200-1000 I.U. daily dose), or
β-carotene (10-25,000 I.U. daily dose) or other naturally
occurring plant antioxidants such as tocopherols, phenolic
compounds, thiols, and ubiquinones can be added to the
25 oral or intravenous formulations of the NO precursor amino
acid(s) or peptides containing the NO precursor amino
acid(s). Alternatively, one may include the active agent
in a nutritional supplement, where other additives may
include vitamins, amino acids other than the NO precursor
30 amino acid(s), or the like, where the subject active agent
will be at least 10 weight %, more usually at least about
25 weight % of the active ingredients.

The administration of the NO precursor amino acid(s)
or its physiologic equivalent in supporting NO can be
35 administered prophylactically to improve vascular
function, serving to enhance vasodilation and to inhibit

16

atherogenesis or restenosis, or therapeutically after
atherogenesis has been initiated. Thus, for example, a
patient who is to undergo balloon angioplasty can have a
regimen of the NO precursor amino acid(s) administered
5   substantially prior to the balloon angioplasty, preferably
at least about a week or substantially longer.
Alternatively, in a patient, the administration of NO
precursor amino acid(s) can begin at any time.
Conveniently, the amino acid composition can be
10 administered by incorporating the appropriate dose in a
prepared food. Types of foods include gelatins, ice
creams, cereals, candies, sugar substitutes, soft drinks,
and the like. Of particular interest is the incorporation
of NO precursor amino acid(s) as a supplement in a food,
15 such as a health bar, e.g. granola, other grains, fruit
bars, such as a date bar, fig bar, apricot bar, or the
like. The amount of NO precursor amino acid(s) or the
equivalent would be about 1 - 25 g per dosage or bar,
preferably about 2 - 15 g.
20   Methods and devices are provided for the
treatment of pathologies associated with vascular injury,
particularly in relation to angioplasty and atherectomy.
Of particular interest is the injury referred to as
restenosis, which results from the migration and
25 proliferation of vascular smooth muscle cells into the
intima of the vessel as well as accretions associated with
the atherosclerosis.
         Instead of oral administration of the NO precursor
amino acid(s), intravascular administration can be
30 employed, particularly where more rapid enhancement of the
nitric oxide level in the vascular system is desired (i.e.
as with acute thrombosis of a critical vessel), so that
combinations of oral and parenteral administrations can be
employed in accordance with the needs of the patient.
35 Furthermore, parenteral administration can allow for the
administration of compounds which would not readily be

17

SUBSTITUTE SHEET (RULE 26)

transported across the mucosa from the gastrointestinal tract into the vascular system.

In a particular application for intramural treatment to prevent restenosis, either L-lysine or L-arginine may
5 be used, individually, or in combination, as well as their biological equivalents, by themselves or in conjunction with other non-amino acid factors. described herein. The method provides introducing into the vessel walls at the site of injury an NO precursor, which results in the
10 enhancement of NO production in the cells at the site of injury. Various delivery systems may be employed which result in the NO forming agent infusing into the vessel wall, and being available to the cells for NO production. Devices which may be employed include drug delivery
15 balloons, e.g. porous, sonophoretic, and iontophoretic balloons, as exemplified by the devices depicted in WO92/11895, WO95/05866 and WO96/08286, as well as such commercial devices as Dispatch® (Scimed). See also Santoian et al., *Cath. Cardiov. Diag.* (1993) 30:348-354;
20 Muller et al., *J. Am. Coll. Cardiol.* (1992) 20:460-466; and Ortiz et al., *Circulation* (1994) 89:1518-1522. The NO precursor agent is introduced in a delivery balloon for transport by a catheter to the site of injury. The balloon may then be expanded under pressure driving the
25 agent from the balloon into the surrounding vessel wall. The amount of agent which is employed may vary depending upon the nature of the agent, the region to be treated, and the loss of the agent from the region. The infusion of the agent is maintained for sufficient time to ensure
30 that the cells and extracellular matrix in the injury region are exposed to the agent, so as to enhance the production of NO by these cells.

A physiologically acceptable medium will be employed, normally an aqueous medium, which may be deionized water,
35 saline, phosphate buffered saline, etc. The amount of the active NO precursor agent will vary depending upon the

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particular agent employed, the other additives present,
etc. Generally, as exemplified by L-arginine, at least
about 50 mg will be present, and not more than about 5 g,
usually at least about 100 mg, and not more than about 2

5   g, frequently at least about 500 mg. The concentration
may be varied widely, generally ranging from about 20-500,
more usually from about 50-250 g/l.

The time for the treatment will usually be at least
about 2 minutes, and not more than about 0.5 hour,

10 generally ranging from about 5-15 minutes. The rate of
introduction will generally range from about 0.05-5
ml/min, depending upon all of the other factors.

The subject methodology is employed with hosts who
have suffered vascular injury, as caused by angioplasty

15 and atherectomies. The time for the administration of the
NO precursor agent may be varied widely, providing a
single administration or multiple administrations over a
relatively short time period in relation to the time of
injury. Generally, treatment may be before, concurrently

20 or after the injury, usually within 2 weeks of the injury,
if before, and not more than about 8 weeks, usually not
more than about 6 weeks, preferably in the range of 0-6
weeks (where 0 intends concurrrently or shortly after the
prior procedure, within 6 hours).

25   For the most part, the patients will be suffering
from various conditions associated with narrowed vessels,
particularly hypercholesterolemia, diabetes, tobacco use
and hypertension. Thus, one will normally be dealing with
vessels which are narrowed to varying degrees as a result

30 of the accumulation of plaque at the vessel wall.

It is found that with one treatment of the NO
precursor agent at or about the time of the injury, before
or shortly thereafter, one can observe enhanced vascular
NO production and reduced intimal thickening, so as to

35 substantially reduce the potential for restenosis.

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As indicated, various delivery devices may be
employed for the delivery of the active agent. Figure 15
illustrates the drug delivery apparatus with the balloon
12 in its inflated state and within an arterial vessel in
5   which the vessel walls are indicated by the reference
numeral 15. During percutaneous transluminal coronary
angioplasty ("PCTA") procedures, the guide wire 10 is
first inserted into the selected artery to a point past
the stenotic lesion. The dilatation catheter including
10 the catheter body 11 and the balloon 12 is then advanced
along the guide wire 10 to the desired position in the
arterial system in which the balloon portion 12 traverses
or crosses the stenotic lesion. The balloon 12 is then
inflated by introducing the NO precursor solution through
15 the balloon lumen 14 into the interior chamber 13 of the
balloon 12. During inflation, the outer surfaces of the
balloon 12 press outwardly against the inner surfaces of
the vessel wall 15 to expand or dilate the vessel in the
area of the stenotic lesion, thus performing the
20 angioplasty portion of the method as well as the
intramural introduction of the NO precursor into the
vessel wall.

The porous balloon may be made from any of the
conventional materials used for this purpose. These
25 include cellulose acetate, polyvinyl chloride,
polysulfone, polyacrylonitrile, polyurethanes, natural and
synthetic elastomers, polyolefins, polyesters,
fluoropolymers, etc. Usually the film thickness will be
in the range of about 10Å to 1μ, with a nominal pore size
30 of about 0.05 to 1μ.

Alternatively, a local drug delivery system may be
employed where the agent is delivered to the vessel wall
by channels that are on the exterior surface of the
balloon. The balloon is placed into the diseased vessel
35 segment as described above. The balloon is then inflated
in the usual manner (using saline, usually containing a

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contrast agent), placing the channels (on the surface of
the balloon) in contact with the vessel wall. The NO
precursor solution is then infused under pressure into the
channels. Perforations in the channels allow the solution
5 to exit and jet into the vessel wall under pressure to
enhance intramural delivery.

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the balloon) in contact with the vessel wall. The NO
15 precursor solution is then infused under pressure into the
channels. Perforations in the channels allow the solution
to exit and jet into the vessel wall under pressure to
enhance intramural delivery.

For oral or intravascular administration, one can
20 provide NO precursor amino acid(s), by itself or in a
polypeptide, or its physiological equivalent in supporting
NO, together with antioxidants or scavengers of oxygen-
derived free radicals (such as sulfhydryl containing
compounds) or compounds that prevent the production of
25 oxygen-derived free radicals (such as superoxide
dismutase), as it is known that oxygen-derived free
radicals (such as superoxide anion) can inactivate nitric
oxide. In addition, one can administer cofactors required
for NO synthase activity, such as calcium or folate. The
30 amounts of each of these co-agents can be determined
empirically, using the assays in the experimental section
to determine NO activity.

The various cofactors that may be used with the NO
precursors will vary in amount in relation to the amount
35 of NO precursor and the effectiveness of the cofactor,
particularly for oral administration. Generally, the

21

cofactors may be present in amounts that would provide daily doses of folate (0.4-10 mg), B_6 (50-250 mg), B_{12} (0.5-1 mg) and/or calcium (250-1000 mg). Usually, where the amount of the NO precursor is greater than about 2g,

5 it may be administered periodically during the day, being administered 2 to 4 times daily. For the most part, the cofactors will be GRAS substances and will be able to be taken at high dosages without adverse effects on the recipient host.

10 The subject compositions will be for the most part administered orally and the dosage may take a variety of forms. The dosage may be tablets, pill, capsules, powders, solutions, dispersions, bars, ice creams, gelatins, and the like, formulated with physiologically

15 acceptable carriers, and optionally stabilizers, colorants, flavoring agents, excipients, tabletting additives, and the like. Depending upon the mode of administration, the amount of active agent may be up to about 25g. For formulations as dietary supplements,

20 individual dosages will generally range from about 0.5 to 5g, more usually from about 1 to 3g of the NO precursor.

Alternatively, one can enhance, either in conjunction with the enhancement of precursors to nitric oxides or independently, components of the nitric oxide metabolic

25 pathway. For example, one can enhance the amount of nitric oxide synthase present in the vessel wall, particularly at the site of lesions. This can be done by local administration to the lesion site or systemically into the vascular system. The synthase can be

30 administered using liposomes, slow release particles, or in the form of a depot, e.g. in collagen, hyaluronic acid, biocompatible gels, vascular stents, or other means, which will provide the desired concentration of the NO synthase at the lesion site.

35 Instead of providing for the enhancement of NO at the physiological site of interest, one can choose to extend

22

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the lifetime of the signal transduced as a result of the presence of nitric oxide. Since cGMP is produced intracellularly as a result of a nitric oxide induced signal, employing agents which result in the production of

5   or extending the lifetime of cGMP can be employed. Illustrative agents include cGMP phosphodiesterase inhibitors or agents which increase the amount of guanylate cyclase.

Alternatively, cells can be genetically engineered to

10 provide for constitutive or inducible expression of one or more genes, which will provide for the desired relaxation response, by expressing NO synthase, or other enzyme or protein which is secreted and acts extracellularly. Thus, expression vectors (viral or plasmid) can be prepared

15 which contain the appropriate gene(s) and which can be introduced into host cells which will then produce high concentrations of nitric oxide or other intermediate in the relaxation pathway. These cells can be introduced at the lesion site or at another site in the host, where the

20 expression will induce the appropriate response as to relaxation, proliferation, etc. The NO synthase or cells expressing the NO synthase can be present as depots by encapsulation and positioning at the site of interest. For example, porous stents can be produced which

25 encapsulate the enzyme or cells to protect the enzyme from degradation or being washed away.

Cultured cells can be transfected with expression vectors containing the NO synthase or other gene ex-vivo and then introduced into the vessel wall, using various

30 intra-arterial or intra-venous catheter delivery systems. Alternatively, techniques of in vivo gene transfer can be employed to transfect vascular cells within the intact vessel in vivo. The gene(s) can be expressed at high constitutive levels or can be linked to an inducible

35 promoter (which can have tissue specificity) to allow for regulation of expression.

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DNA constructs are prepared, where the appropriate gene, e.g. a NO synthase gene, is joined to an appropriate promoter, either with its native termination region or a different termination region, which can provide for
5   enhanced stability of the messenger RNA. Constitutive promoters of particular interest will come from viruses, such as Simian virus, papilloma virus, adenovirus, HIV, Rous sarcoma virus, cytomegalovirus or the like, where the promoters include promoters for early or late genes, or
10   long terminal repeats. Endogenous promoters can include the β-actin promoter, or cell-type specific promoters.

A construct is prepared in accordance with conventional techniques, the various DNA fragments being introduced into an appropriate plasmid or viral vector,
15   normally a vector capable of replication in a bacterial and/or eucaryotic host. Normally, the vector will include a marker, which allows for selection of cells carrying the vector, e.g. antibiotic resistance. The vector will normally also include an origin which is functional in the
20   host for replication. Other functional elements can also be present in the vector.

Once the vector has been prepared and replicated, it can then be used for introduction into host cells. The plasmid vector construct can be further modified by being
25   joined to viral elements which allow for ease of transfection, can provide a marker for selection, e.g. antibiotic resistance, or other functional elements. Introduction of the plasmid vector construct into the host cells can be achieved by calcium phosphate precipitated
30   DNA, transfection, electroporation, fusion, lipofection, viral capsid-mediated transfer, or the like. Alternatively, the expression construct within viral vectors can be introduced by standard infection techniques. For somatic cell gene therapy, autologous
35   cells will generally be employed, although in some instances allogeneic cells or recombinantly modified cells

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can be employed. Usually the cells employed for genetic
modification will be mature endothelial or vascular smooth
muscle cells. Occasionally, the cells employed for
genetic modification will be progenitor cells,
5   particularly early progenitor cells. For example,
myoblasts can be employed for muscle cells or
hematopoietic stem cells or high proliferative potential
cells can be employed for lymphoid and/or myelomonocytic
cells.

10   Depending upon the nature of the cells, they can be
injected into tissue of the same or different cellular
nature, they can be injected into the vascular system,
where they may remain as mobile cells or home to a
particular site (i.e. the lesion). For the NO synthase
15 gene, the number of cells which are administered will
depend upon the nature of the cells, the level of
production of the NO synthase, the desired level of NO
synthase in the host vascular system, at the lesion site,
or the like, whether the enhanced level of NO synthase is
20 the only treatment or is used in conjunction with other
components of the nitric oxide synthetic pathway, and the
like. Therefore, the particular number of cells to be
employed will be determined empirically in accordance with
the requirements of the particular patient.

25   These cells can also be introduced into the
circulation by first growing them on the surface of
standard vascular graft material (i.e. Dacron or
polytetrafluoroethylene grafts) or other synthetic
vascular conduits or vascular bioprostheses.

30   Alternatively, one can use viral vectors, which are
capable of infecting cells *in vivo*, such as adenovirus or
retroviruses. In this case, the viral vector containing
the NO synthase gene or other gene involved with the
relaxation pathway will be administered directly to the
35 site of interest, where it will enter into a number of
cells and become integrated into the cell genome. Thus,

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one can titer the desired level of nitric oxide synthase which is secreted or other protein which is expressed, by providing for one or more administrations of the virus, thus incrementally increasing the amount of synthase which

5 is secreted or other protein which is produced.

Alternatively, one can use modified liposomes as a vehicle for endovascular administration of the vector containing the NO synthase or other gene. One such modified liposome technique involves mixing the liposomes

10 with the vector containing NO synthase. Once the gene expression construct-containing vector is incorporated into the liposome, the liposomes are coated with a protein (e.g. the viral coat protein of the Hemagglutinating Virus of Japan) that increases the affinity of the liposome for

15 the vessel wall.

In some situations, the NO synthase or other gene in the relaxation pathway can be co-transfected with an artificial gene encoding an lysine and/or arginine rich polypeptide susceptible to proteolytic cleavage as an

20 intracellular source of L-lysine and/or L-arginine. In other situations, the NO synthase or other gene can be co-transfected with the superoxide dismutase gene, so as to inhibit the degradation of the nitric oxide.

In some situations, acute treatment may be involved,

25 requiring one or a few administrations. This will normally be associated with compounds which can act as nitric oxide precursors and are other than naturally occurring compounds or are compounds which can be added with naturally occurring compounds to enhance the rate of

30 formation of nitric oxide. Thus, one can provide for acute administration of NO precursor amino acid(s) and superoxide dismutase to increase the nitric oxide concentration over a restricted period of time. These administrations can be independent of or in conjunction

35 with long term regimens.

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The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

5   Example 1. **Anti-atherogenic effects of oral arginine:**

Study design: (See, Cooke et al., 1992, *supra*) Male New Zealand white rabbits (n=49) were assigned to one of three treatment groups: 10 were fed with normal rabbit
10   chow for ten weeks (Control); 19 received chow enriched with 1% cholesterol (Chol); and 20 received a 1% cholesterol diet supplemented with 2.25% L-arginine hydrochloride in the drinking water (Arg.). Following ten weeks of the dietary intervention, animals were lightly
15   sedated and the central ear artery cannulated for measurement of intra-arterial blood pressure, followed by collection of blood samples for serum chemistries and plasma arginine. Subsequently the animals were sacrificed and the left main coronary artery and the thoracic aorta
20   were harvested for studies of vascular reactivity and histomorphometry. In some animals, blood was collected for studies of platelet and monocyte reactivity.

Results: Biochemical and physiological measurements. Hypercholesterolemic animals maintained on oral L-arginine
25   supplementation (Arg) experienced a twofold elevation in plasma arginine levels in comparison to animals on a normal (Control) or 1% cholesterol (Chol) diet alone; the elevation in plasma arginine was maintained throughout the course of the study. Serum cholesterol measurements were
30   elevated equally in both groups receiving the 1% cholesterol diet [50±6 vs. 1629±422 vs. 1852±356 mg/dl respectively for Control (=10), Chol (=13), and Arg (=14)]. There were no significant differences in hemodynamic measurements between groups.
35   **Organ chamber studies of isolated vessels:** For NO-independent responses, there were no differences between

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the treatment groups in maximal response or sensitivity to norepinephrine (a vasoconstrictor), or to nitroglycerin (a nitrovasodilator). By contrast, NO-dependent relaxations were attenuated in vessels harvested from

5 hypercholesterolemic animals with a reduction in the maximal response to acetylcholine. In comparison, vessels harvested from hypercholesterolemic animals receiving L-arginine supplementation had improved NO-dependent relaxation to acetylcholine. In a separate study, the

10 effect of chronic arginine supplementation to improve NO-dependent relaxation was confirmed in the hypercholesterolemic rabbit abdominal aorta.

Histomorphometric studies (planimetry of EVG-stained
15 **sections):** A blinded histomorphometric analysis revealed that medial cross-sectional areas of thoracic aortae were not different between the groups. By contrast, the intimal cross-sectional area (i.e. amount of atherosclerotic plaque) of vessels from

20 hypercholesterolemic animals receiving L-arginine supplementation was reduced in comparison to those from animals receiving cholesterol diet alone. In the Arg animals the reduction in the intimal lesion was most pronounced in the ascending thoracic aorta and left main

25 coronary artery. In the left main coronary artery of hypercholesterolemic animals receiving arginine, essentially no atherosclerotic plaque developed.

Changes in lesion surface area: In a second series of studies, the extent of the thoracic aorta involved by

30 lesions was examined. In hypercholesterolemic rabbits receiving vehicle (n=6) or L-arginine supplement (n=6), thoracic aortae (from left subclavian artery to diaphragm) were harvested after ten weeks of treatment, bisected longitudinally, and stained with oil-red O. Vessels were

35 photographed and vessel and lesion surface area determined by planimetry. Approximately 40% of the total surface

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area was covered with plaque in thoracic aortae from hypercholesterolemic animals receiving vehicle, whereas in thoracic aortae from arginine-treated hypercholesterolemic animals, less than 10% of the surface area was covered
5 with plaque (Figure 1).

To summarize, dietary arginine supplementation increases plasma arginine levels, but does not alter serum cholesterol. This is associated with significant improvement in NO-dependent vasodilation as judged by
10 bioassay. Finally, the improvement in NO-dependent vasodilation is associated with reduction in thickness and area of the lesions in vessels from hypercholesterolemic animals.

15   Example 2. Inhibition of platelet aggregation by oral L-arginine: The effect of L-arginine supplementation on platelet reactivity in rabbits that had normal chow (Control; n = 6), a 1% cholesterol diet (Chol; n = 5), or a 1% cholesterol diet supplemented with oral arginine
20 (Arg; n = 6), as detailed above, was examined. Arterial blood obtained after central ear artery cannulation was anticoagulated with 13mM sodium citrate. Platelet-rich suspension was prepared by washing platelets in calcium-free Krebs-Henseleit solution and resuspending them in
25 Tyrode's solution with albumin. Aggregation was initiated by addition of adenosine diphosphate and monitored by standard nephelometric techniques. In platelets derived from Chol animals, aggregation was not different in rate or maximum extent in comparison to platelets from Control
30 animals (A, in Figure 2). By contrast, aggregation of platelets from Arg animals was reduced by 50% (B, in Figure 2).

This reduction in platelet aggregation was associated with a two-fold greater cGMP content in aggregated
35 platelets from arginine-treated animals. The reduction of platelet reactivity could be reversed by administration of

29

SUBSTITUTE SHEET (RULE 26)

N-methylarginine (10^{-4}M) in vitro (C, in Figure 2).
Therefore, the anti-platelet effect of chronic oral
arginine administration can be credited to an increased
synthesis of endogenous NO. Furthermore, NO synthesis may
5 be induced in both the platelets and the endothelium.

Example 3. Inhibition of monocyte adherence:
A. Functional Binding Assay: To determine if oral
arginine supplementation affects monocyte adherence, blood
10 was collected from rabbits fed normal chow (=6) a 1%
cholesterol diet (=6), or a 1% cholesterol diet
supplemented with L-arginine (=6), as described above.
Mononuclear cells were purified from blood by Ficoll-paque
density gradient centrifugation.   In these preliminary
15 studies, adhesion was examined of blood leukocytes to a
transformed endothelial cell line, bEnd3 (mouse brain-
derived polyoma middle T antigen transformed endothelial
cells). The Bend3 cells display the morphology of
endothelial cells, and like human endothelial cells are
20 capable of uptake of acetylated low-density lipoprotein
and express adhesion molecules in a cytokine-regulatable
fashion. Cultured cells were grown to confluence in 0.5
cm^2 Lab-Tek chamber slides (MilesScientific) and treated
with control medium or with LPS (1 mg/ml) or TNFα (25
25 U/ml) for 18 hours. Cultures were washed with fresh assay
buffer, and low, medium, or high concentrations of
leukocytes (0.75, 1.5, or 3 x 10^5 cells/ml, respectively)
were added per well. Following a 30-minute incubation on
a rocking platform at room temperature to allow binding,
30 the slides were inverted and immersed in buffer containing
2% (v/v) glutaraldehyde, such that non-adherent cells were
lost and adherent cells were fixed to the monolayer. The
adherent mononuclear cells were enumerated using video-
light microscopy.
35   Monocytes from hypercholesterolemic animals (Chol)
exhibited greater adherence, consistent with observation

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by others, that monocytes from hypercholesterolemic cats
or humans exhibit greater adherence to cultured
endothelial cells. (deGruijter et al. (1991) *Metabol.
Clin. Exp.* 40:1119-1121; Fan et al. (1991) *Virchows Arch.*
5   *B Cell Pathol.* 61:19-27).

        In comparison to monocytes derived from vehicle-
treated hypercholesterolemic animals (Chol), those from
arginine-treated hypercholesterolemic animals (Arg) were
much less adherent. This data shows that the arginine
10 treatment inhibits adhesion of monocytes to the
endothelium, which is the first observable event in
atherogenesis.

        Example 4. Dietary L-Arginine Inhibits the Enhanced
15 Endothelial-Monocyte Interaction In Hypercholesterolemia
        The earliest observable abnormality of the vessel
wall in hypercholesterolemic animals is enhanced monocyte
adherence to the endothelium, which occurs within one week
of a high cholesterol diet. This event is thought to be
20 mediated by the surface expression of endothelial adhesion
molecules and chemotactic proteins induced by
hypercholesterolemia.

        Another endothelial alteration that occurs in
parallel is a reduced activity of nitric oxide (i.e., NO),
25 derived from metabolism of L-arginine. As shown above
chronic dietary supplementation with L-arginine restores
NO-dependent vasodilatation in hypercholesterolemic
rabbits, and that this improvement in NO activity is
associated with a striking anti-atherogenic effect. In
30 the following study was tested the hypothesis that the
anti-atherogenic effect of dietary arginine was mediated
by endothelial derived NO which inhibits monocyte-
endothelial cell interaction.

        Methods. *Animals.* Male New Zealand White rabbits
35 were pair fed, receiving one of the following dietary
interventions for two weeks: normal rabbit chow (Cont,

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n=7); rabbit chow enriched with 1% cholesterol (Chol, n=7); or 1% cholesterol chow supplemented with 2.25% L-arginine HCl in the drinking water (Arg, n=7) *ad libitum* throughout the course of the study. In a second series of

5   studies designed to further explore the role of endogenous NO on monocyte-endothelial cell interaction, another group of animals were pair fed, receiving a normal rabbit diet supplemented with either vehicle control (N=5) or the NO synthase antagonist, nitro-L-arginine (L-NA, 10 mg/100 ml;

10 n=5), administered in the drinking water *ad libitum* throughout the course of the study (for an average daily dose of 13.5 mg/kg/day). In a third series of experiments animals received a normal diet and either vehicle (n=4), L-NA (13.5 mg/kg/day; n=4), or L-NA and hydralazine (n=4)

15 added to the drinking water for two weeks. At this dose, hydralazine (5 mg/kg/day) reversed the increase in blood pressure induced by L-NA. One day before sacrifice (after 2 weeks of dietary intervention), animals were lightly sedated and the central ear artery was cannulated for

20 collection of blood samples.

   Mononuclear cell culture and isolation. Murine monocytoid cells, WEHI 78/24 cells were grown in Dulbecco's Modified Eagle's Medium supplemented 10% fetal calf serum (vol/vol) and were kept in an atmosphere of 5%

25 CO_2/95% air. Prior to binding studies, mononuclear cells were fluorescently labeled with TRITC (3 μg/ml). To confirm the results using WEHI cells, in some studies binding studies were performed in parallel using rabbit mononuclear cells. Mononuclear cells were isolated from

30 fresh whole blood of Control rabbits before sacrifice.

   Preparation of aortic endothelium and binding assay. After 2 weeks of the dietary intervention, the thoracic aortae were removed and placed in cold, oxygenated saline. A 15 mm segment of thoracic aorta was excised from a point

35 immediately distal to the left subclavian artery to the mid-thoracic aorta. The segments were then carefully

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opened longitudinally and placed into culture dishes
containing HBSS medium. Aortic strips were fixed to the
culture dish using 25 gauge needles so as to expose the
endothelial surface to the medium. Culture dishes were
5 then placed on a rocking platform at room temperature.

After 10 minutes the HBSS medium was replaced by
binding medium containing WEHI cells. The aortic strips
were incubated with the mononuclear cells for 30 minutes.
The medium was then replaced by fresh binding medium
10 without cells to remove non-adherent cells. The aortic
segments were then removed and placed on a glass slide,
and adherent cells counted under epifluorescent microscopy
from at least 30 sites on each segment.

Results. *Monocyte adhesion to rabbit aortic
15 endothelium.* Exposure of WEHI 78/24 cells to normal
rabbit aortic endothelium results in a minimal cell
binding in this *ex vivo* adhesion assay. However, when
WEHI cells were incubated with aortic endothelium from
hypercholesterolemic animals (Chol; n=7), cell binding
20 was enhanced 3-fold in comparison to Cont (n=7). The
increased cell binding manifested by aortic endothelium of
hypercholesterolemic animals was significantly attenuated
by L-arginine supplementation (n=7). (Figure 3) Similar
results were achieved when adhesion assays were performed
25 in parallel with mononuclear cells that were freshly
isolated from Cont animals (n=2) in each of the three
groups.

*Effect of chronic NO synthase inhibition on
endothelial adhesiveness.* To further investigate the role
30 of endothelium-derived NO in modulating endothelial-
monocyte interaction, an additional series of binding
studies were performed using thoracic aorta from animals
that received regular chow supplemented with vehicle (n=5)
or the NO synthase inhibitor, L-NA (n=5). The adhesion of
35 WEHI cells was markedly increased when incubated with
aortic endothelium from L-NA animals compared to control

33

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endothelium. This effect could not be attributed to hypertension caused by L-NA since concomitant administration of hydralazine normalized blood pressure but did not reverse the augmentation of cell binding

5   induced by L-NA.

In a separate series of studies it was confirmed that chronic administration of L-NA (the inhibitor of NO synthase) significantly inhibited generation and release of NO from the vessel wall (as measured by

10   chemiluminescence), compared to vessels from animals treated with vehicle or arginine.

The salient findings of this investigation are: 1) monocyte binding to the endothelium *ex vivo* is increased in vessels from hypercholesterolemic animals; 2) this

15   increase in monocyte binding is attenuated in hypercholesterolemic animals treated chronically with the NO precursor L-arginine; 3) monocyte binding to the endothelium is increased in vessels from normocholesterolemic animals treated with the NO synthase

20   antagonist L-nitro-arginine; and 4) this effect of NO synthase antagonism was not reversed by administration of hydralazine in doses sufficient to normalize blood pressure. These findings are consistent with the hypothesis that NO inhibits monocyte-endothelial cell

25   interaction.

To conclude, an *ex vivo* model of monocyte binding has been used to study the increase in endothelial adhesiveness induced by hypercholesterolemia. Endothelial adhesiveness is attenuated by oral administration of the

30   NO precursor L-arginine is shown. Conversely, inhibition of NO synthase activity by oral administration of nitro-arginine strikingly increases endothelial affinity for monocytes *ex vivo*. The data are consistent with NO being an endogenous anti-atherogenic molecule.

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Example 5. Oral Arginine causes regression of
atherosclerosis in hypercholesterolemic rabbits: Our
previous work demonstrated that oral arginine could
prevent the development of plaque in hypercholesterolemic
5   animals but it was not known if pre-existing plaque could
be affected by arginine treatment. This is clinically
important if arginine is to be useful in the treatment of
pre-existing atherosclerosis in humans. Accordingly, New
Zealand white rabbits (n=85) received normal chow or 0.5%
10   cholesterol chow for 10 weeks. Subsequently, half of the
hypercholesterolemic rabbits were given 2.25% (W/V) L-
arginine in their drinking water. Thoracic aortae were
harvested at weeks 10, 14, 18, or 23. Rings of aorta were
used to assess NO-dependent vasodilation to acetylcholine
15   (ACh). Maximal relaxation to ACh in the
hypercholesterolemic rabbits receiving vehicle (CHOL)
became progressively attenuated from 53.4% (at week 10) to
17.4% (by week 23). Planimetry of the luminal surface of
the aortae from CHOL animals revealed a progressive
20   increase in plaque area from 30.3% (at week 10) to 56.5%
(by week 23) of the total surface of the thoracic aorta.
By contrast, hypercholesterolemic animals receiving
arginine (ARG) manifested improved endothelium-dependent
relaxation associated with a reduction of plaque area at
25   14 and 18 weeks. Lesion surface area in all arginine
treated hypercholesterolemic animals (weeks 14-23) was
significantly reduced in comparison to vehicle-treated
hypercholesterolemic animals (Figure 4). The arginine-
induced improvement in endothelium-dependent relaxation
30   was associated with an increased generation of vascular
NO, and a reduced generation of vascular superoxide anion.
By 23 weeks, 3 of 7 ARG animals had persistent improvement
in NO-dependent vasodilation and exhibited a further
reduction of plaque area to 5.4%
35   Conclusions: hypercholesterolemia induces a
progressive loss of NO-dependent vasodilation associated

35

with progressive intimal lesion formation. Administration
of L-arginine to animals with pre-existing intimal lesions
augments vascular NO elaboration, reduces superoxide anion
generation, and is associated with a reduction in plaque
5   area. This is the first demonstration that restoration of
NO activity can induce regression of pre-existing intimal
lesions, and provides evidence that L-arginine therapy may
be of potential clinical benefit.

10   Example 6. Oral arginine administration restores
vascular NO activity and inhibits myointimal hyperplasia
after balloon injury in hypercholesterolemic rabbits.
          Purpose. The purpose of this study was to determine
if the alterations in vascular function and structure
15   following balloon angioplasty in hypercholesterolemic
rabbits could be inhibited by restoration of endogenous
nitric oxide (NO) activity.
          Methods. Twenty-eight New Zealand white rabbits were
randomized into one of three dietary groups and received
20   either normal rabbit chow, 0.5% cholesterol diet, or 0.5%
cholesterol diet plus L-arginine hydrochloride (2.25% W/V)
in the drinking water. After six weeks of dietary
intervention, the left iliac artery of each animal was
subjected to a balloon angioplasty. Four weeks later, the
25   iliac arteries were harvested for vascular reactivity
studies and immunohistochemistry.
          Results. The bioassay studies indicated that
endothelium-derived NO activity was inhibited in
hypercholesterolemic animals in comparison to
30   normocholesterolemic animals. The administration of
arginine partially restored endothelium-derived NO
activity. Balloon angioplasty induced intimal thickening
which was largely composed of vascular smooth muscle cells
and extracellular matrix. In the setting of
35   hypercholesterolemia, vascular injury induced an exuberant
myointimal lesion that was augmented by the accumulation

36

of lipid-laden macrophages. Administration of L-arginine
induced a quantitative as well as qualitative change in
the lesion. Dietary arginine reduced intimal thickening
in the injured vessels of hypercholesterolemic animals,
5 and substantially inhibited the accumulation of
macrophages in the lesion (Figure 5).

Conclusions. We report that the lesions induced by
balloon angioplasty in hypercholesterolemic animals are
markedly reduced by oral administration of arginine.
10 Moreover, we find that the nature of the lesion is
altered, with a striking reduction in the percentage of
macrophages comprising the lesion. Hypercholesterolemia
induces an endothelial vasodilator dysfunction in the
rabbit iliac artery that is reversible by chronic oral
15 administration of arginine.

**Example 7. Nitric oxide regulates monocyte
chemotactic protein-1.** Our previous studies had
established that oral arginine administration could
20 enhance vascular NO synthesis. This increase in vascular
NO synthesis was associated with inhibition of monocyte
adherence and accumulation in the vessel wall (thereby
reducing the progression, and even inducing regression, of
plaque). The question remained: "How does vascular nitric
25 oxide inhibit monocyte adherence and accumulation in the
vessel wall?"

Monocyte chemotactic protein-1 (MCP-1) is a 76-amino
acid chemokine thought to be the major chemotactic factor
for monocytes (chemotactic factors are proteins that
30 attract white blood cells). We hypothesized that the
anti-atherogenic effect of NO may be due in part to its
inhibition of MCP-1 expression.

Methods and Results. Smooth muscle cells (SMC) were
isolated from normal rabbit aortae by explant method.
35 Cells were then exposed to oxidized LDL (30 μg/ml) (which
is known to induce vascular cells to synthesize MCP-1).

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The expression of MCP-1 in SMC was associated with an increased generation of superoxide anion by the SMC, and increased activity of the transcriptional protein NFκB. All of these effects of oxidized LDL cholesterol were

5 reduced by previous exposure of the SMC to the NO-donor DETA-NONOate (100 μM) ($p<0.05$). To determine if NO exerted its effect at a transcriptional level, SMC and COS cells were transfected with a 400 bp fragment of the MCP-1 promoter. Enhanced promoter activity by oxLDL was

10 inhibited by DETA-NO.

To investigate the role of endogenous NO in the regulation of MCP-1 in vivo, NZW rabbits were fed normal chow, normal chow plus nitro-L-arginine (L-NA) (to inhibit vascular NO synthesis), high cholesterol diet (Chol), or

15 high cholesterol diet supplemented with L-arginine (Arg) (to enhance NO synthesis). After two weeks, thoracic aortae were harvested and total RNA was isolated. Northern analysis demonstrated increased expression of MCP-1 in Chol and L-NA aortae; this expression was

20 decreased in aortae from Arg animals. These studies indicate that the anti-atherogenic effect of NO may be mediated in part by its inhibition of MCP-1 expression. NO inhibits the generation of superoxide anion by the vascular cells and thereby turns off an oxidant-responsive

25 transcriptional pathway (i.e. NFκB-mediated transcription) activating MCP-1 expression.

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Example 8. **Nitric Oxide inhibits the expression of
an endothelial adhesion molecule known to be involved in
atherosclerosis.**

Vascular cell adhesion molecule (VCAM-1) is an
5   endothelial adhesion molecule that binds monocytes. This
molecule is expressed by the endothelium of
hypercholesterolemic animals, and is expressed by
endothelial cells overlying plaque in animals and humans.
This adhesion molecule is believed to participate in
10   monocyte adherence and accumulation in the vessel wall
during the development of plaque. Other workers have
shown that the expression of this molecule is regulated by
an oxidant-responsive transcriptional pathway mediated by
the transcriptional factor NFκB. Endothelial cells
15   exposed to oxidized LDL cholesterol (or cytokines like
TNF-α) begin to generate superoxide anion. Superoxide
anion turns on oxidant-responsive transcription leading to
the expression of VCAM-1 and MCP-1 (and probably other
genes that participate in atherosclerosis). Our data
20   indicates that NO inhibits the generation of superoxide
anion, thereby turning off these oxidant-responsive
transcriptional pathways.

Methods and Results: Confluent monolayers of human
aortic endothelial cells (HAEC) were exposed to static or
25   fluid flow conditions for 4 hours (fluid flow stimulates
the production of endogenous nitric oxide). Medium was
then replaced and cells were then incubated with native
LDL (50 μg/ml), oxidized LDL (30 μg/ml), or LPS (10 ng/ml)
+ TNF-α (10 U/ml) for an additional 4 hours. Functional
30   binding assays utilizing THP-1 monocytes were then
performed. Superoxide production by HAECs was monitored
by lucigenin chemiluminescence and expression of the
adhesion molecules VCAM-1 and ICAM-1 was quantitated by
flow cytometry. Whereas native LDL had little effect,
35   incubation with either oxLDL or LPS/TNF significantly
increased superoxide production, NF-κB activity, VCAM-1

expression and endothelial adhesiveness for monocytes.
Previous exposure to fluid flow inhibited endothelial
adhesiveness for monocytes (Figure 6) and the other
sequelae of exposure to cytokines or oxidized lipoprotein.
5   The effect of fluid flow was due to shear-induced release
of nitric oxide since coincubation with L-nitro-arginine
completely abolished these effects of flow. Furthermore,
the NO donor PAPA-NONOate mimicked the effects of flow.
    Conclusions. Previous exposure to fluid flow
10 decreased cytokine or lipoprotein-stimulated endothelial
cell superoxide production, VCAM-1 expression and monocyte
binding; the effects of flow are due at least in part to
nitric oxide. NO participates in the regulation of the
endothelial generation of superoxide anion and thereby
15 inhibits oxidant-responsive transcription of genes (i.e.
VCAM-1 and MCP-1) that are involved in atherogenesis.

    Example 9. **Transfection of the gene encoding NO
synthase increases NO generation and inhibits monocyte**
20 **adherence.**

    The following experiment was done to determine if
transfer of the gene encoding NO synthase (the enzyme that
produces NO) could increase generation of nitric oxide and
thereby inhibit monocyte adherence. Cultured endothelial
25 cells   (bEnd-3; a murine endothelial cell line) were
transfected with a plasmid construct encoding the NO
synthase gene, using lipofectamine liposomal technique.
Forty-eight hours later, generation of nitric oxide was
measured using chemiluminescence. Nitric oxide generation
30 was increased 2-fold in cells transfected with the NO
synthase construct (but not in cells transfected with a
control construct). In parallel, binding assays were
performed using a murine monocytoid cell line. The
binding of monocytoid cells to the endothelial cells was
35 reduced by 30% in those cells transfected with the NO
synthase construct.

40

Conclusion: endothelial cells transfected with a plasmid construct containing the NO synthase gene were able to elaborate more nitric oxide. The increased elaboration of nitric oxide was associated with an

5 inhibition of monocyte binding to the endothelial cells.

Example 10. **Effect of NO synthase expression on proliferation of vascular smooth muscle cells:** Cultured rat aortic vascular smooth muscle cells under confluent

10 quiescent conditions were studied. An efficient viral coat protein-mediated DNA transfer method was employed to transfect the cells with the NO synthase gene driven by the β-actin promoter and CMV enhancer. This resulted in increased NO synthase activity (as measured by the

15 arginine-to-citrulline conversion assay) in comparison to control vector transfected cells. Transfection of the NO synthase gene completely abolished serum-stimulated DNA synthesis compared to control vector transfection. These results indicated that increased expression of NO synthase

20 (associated with increased production of NO) inhibits excessive proliferation of vascular smooth muscle cells. This inhibition can be correlated with treatment of atherosclerosis and restenosis.

25 **Example 11.** **Gene Therapy Using NO Synthase cDNA Prevents Restenosis**
The study above indicated that NO inhibits proliferation of vascular smooth muscle cells. In atherogenesis and restenosis, excessive proliferation of

30 vascular smooth muscle cells contributes to lesion formation. Injury to the endothelium in atherosclerosis and after catheter interventions apparently reduces or removes the salutary influence of NO. The following study shows delivery of the gene for NO synthase to the vessel

35 wall inhibits lesion formation.

41

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A plasmid construct encoding the cDNA of endothelial-
type NO synthase (EC-NOS) was synthesized. A full length
cDNA encoding for EC-NOS was inserted into the EcoRI site
of the pUCcaggs expression vector. Balloon angioplasties

5   of the carotid artery in Sprague-Dawley rats were
performed and HVJ-liposomes with plasmids encoding EC-NOS
cDNA infused, or plasmids lacking EC-NOS cDNA (control
vector) infused. After 4 days to 2 weeks, the rats were
sacrificed and the carotid arteries harvested for: 1)

10   histomorphometry; 2) measurement of DNA synthesis; and 3)
ex vivo determination of NO synthesis and release by
bioassay and by chemiluminescence.

Results. Morphometric measurements 2 weeks after
injury revealed a significant (68%) reduction of intimal

15   lesion thickness in EC-NOS treated (Inj + NOS) in
comparison to control vector treated (Inj + CV) or
untreated (Inj) injured vessels. (Figure 7) Measurements
of DNA synthesis were performed four days after injury
using bromodeoxyuridine. EC-NOS transfection

20   significantly limited bromodeoxyuridine incorporation (by
25%) in comparison to control vector treated or untreated
injured vessels. Vessel segments were studied ex vivo
using organ chamber technique to bioassay for NO release.
Calcium ionophore increases intracellular calcium and

25   activates NO synthase to produce NO. Calcium ionophore
induced relaxations in injured carotid arteries
transfected with control vector that were only 15% of
uninjured vessels. Injured arteries that had been
transfected with EC-NOS relaxed to a much greater degree,

30   approximately 50% of that observed in uninjured vessels.
Direct measurement of NO (by chemiluminescence) released
into the medium revealed that NO released by injured
tissues (transfected with the control vector) was only 20%
of that released by normal uninjured tissues. By

35   contrast, injured tissues transfected with EC-NOS released
more NO (about 75% of normal).

To conclude, balloon angioplasty of the rat carotid artery removes the endothelial source of NO, induces excessive vascular smooth muscle DNA synthesis and proliferation, resulting in an intimal lesion

5   (restenosis). Transfection of the vessel with EC-NOS at the time of balloon injury partially restores NO production by the vessel, and this is associated with reduced DNA synthesis and vascular smooth muscle proliferation, thereby reducing lesion formation. These

10 results are consistent with the conclusion that NO is an endogenous anti-atherogenic molecule.

    Example 12. Local application of L-arginine to the vessel wall inhibits myointimal hyperplasia:

15   The previous studies revealed that oral administration of arginine could enhance vascular NO activity and inhibit lesion formation induced by a high cholesterol diet and/or vascular injury (with balloon angioplasty). To determine if intraluminal application of

20 arginine to the vessel wall at the time of balloon angioplasty could inhibit lesion formation, the following study was performed. Rabbits (n=7) were fed a 1% cholesterol diet. After one week, angioplasty of the iliac arteries was performed. After angioplasty of one

25 iliac artery, a local infusion catheter was used to expose the injured area to a high concentration of arginine (6 mM). The other iliac artery was subjected to balloon angioplasty, but not treated with a local infusion. After four weeks, the vessels were harvested, and segments of

30 the arteries processed for histomorphometry. Initial thickening in the arginine-treated vessels was significantly reduced (Figure 8). This study indicates that the local intraluminal application of high doses of arginine can reduce myointimal hyperplasia after vascular

35 injury.

Example 13. Exclusion of the Effect of Enhanced Nitrogen or Caloric Balance as Causing the Observed Results.

To exclude an effect of L-arginine on nitrogen or
5   caloric balance as the cause of these results, six animals received 1% cholesterol diet supplemented by additional methionine to increase the dietary methionine six-fold. At ten weeks animals were sacrificed for studies of platelet and vascular reactivity, and histomorphometry.
10   Endothelium-dependent relaxation, platelet aggregation and intimal thickness were not different from those of animals fed 1% cholesterol diet alone. These results reveal that another amino acid, methionine (which is not a precursor of NO) does not mimic the effect of the amino acid
15   L-arginine. Therefore it seems likely that the effect of L-arginine is due to its metabolism to nitric oxide, rather than some other effect of amino acid administration (i.e. change in nitrogen or caloric balance).

20   **Example 14. L-lysine enhances vascular NO activity and inhibits atherogenesis.**

L-lysine is a basic amino acid like L-arginine, but is not known to be metabolized by NO synthase to NO. Therefore, the following results were unexpected. New
25   Zealand white rabbits were fed a normal or high cholesterol chow (n=18). Half of the animals on the cholesterol diet also received oral L-lysine. After ten weeks, the thoracic aortae were harvested and bioassayed for vascular NO synthesis, and histomorphometry to assess
30   lesion formation was performed as described above. The administration of L-lysine was just as effective as L-arginine to increase vascular NO activity in the hypercholesterolemic animals as assessed by endothelium-dependent vasorelaxation. (Figure 9) The improvement in
35   vascular NO activity was associated with a marked reduction in vascular lesion formation.

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This study revealed the unexpected result that L-lysine can enhance vascular NO activity and inhibit atherosclerosis.

5   Example 15.   Oral L-arginine normalizes monocyte adhesiveness in hypercholesterolemic humans

Adherence of monocytes to the endothelium is the first observable event in the development of atherosclerosis. We hypothesized that chronic oral
10   administration of L-arginine to hypercholesterolemic humans would enhance the generation of endothelium-derived NO, and thereby inhibit the interaction of monocytes with the endothelium. In this investigation we have developed a reproducible assay for the binding of human monocytes to
15   cultured endothelial cells, and we have examined the effect of hypercholesterolemia and L-arginine treatment on this interaction.

The control subject population in this study included 12 normal volunteers, (10 males and 2 females), with an
20   average age of 37 ± 2 yrs. Normalcy was determined by a careful history, physical examination, and laboratory analysis to exclude individuals with hematologic, renal, or hepatic dysfunction or clinically evident atherosclerosis. There were 20 patients (10 males and 10
25   females) with hypercholesterolemia as defined by a total plasma cholesterol greater than 240 mg/dl and a LDL cholesterol level greater than 160 mg/dl. These individuals had an average age of 51 ± 2 yrs . None of the subjects were taking diuretics, vasoactive
30   medications, antiplatelet or hypolipidemic medications. This study was approved by the Stanford University Administrative Panel on Human Subjects in Medical Research and each subject gave written informed consent before entry into the study. Blood was drawn from each subject
35   in the postabsorptive state.

45

We isolated human monocytes from citrated venous blood. The blood was centrifuged and the buffy coat removed and resuspended with HBSS. The suspension was then carefully layered onto a cushion of 1.068-d

5 Histopaque, and centrifuged. After centrifugation, the monocytes were aspirated.

We used the transformed endothelial cell (EC) line, bEnd3 to examine monocyte-endothelial binding *ex vivo*. The bEnd3 cells express endothelial adhesion molecules and

10 bind monocytes in a cytokine-inducible fashion with kinetics similar to those observed with human umbilical vein endothelium. Monocytes were added to the wells containing the endothelial monolayers to reach a final cell number of 3×10^6/ml. In some studies, monocytes

15 were exposed *in vitro* for 30 minutes to sodium nitroprusside (an NO donor) prior to the binding assay.

The six-well plates were transferred to a rocking platform and rocked for 30 minutes at room temperature. After 30 minutes, the cell suspension was aspirated from

20 each well and wells were then rinsed with binding buffer to remove non-adherent monocytes. Videomicroscopic counting of adherent cells was performed using a computer aided image analysis system.

Results. Oral administration of L-arginine (7 g

25 daily for 2 weeks) to hypercholesterolemic humans increased plasma arginine values by 60% (from 79±10 to 128±12 mM; n=7), whereas L-arginine values in the placebo-treated (n=3) and normocholesterolemic (n=6) groups remained unchanged. The administration of oral L-arginine

30 had no effect on any of the biochemical or hematologic parameters and was well tolerated. Oral L-arginine did not lower total cholesterol or LDL cholesterol. Two patients dropped out of the study; one because he did not want to take the pills, and one because of reactivation of

35 oral herpes during the study.

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The results of the adhesion assays were highly
reproducible. Monocytes derived from hypercholesterolemic
individuals demonstrated a 50 ± 8 % increase in bound
cells/hpf in comparison to cells from normal individuals
5   ($p<0.0001$). The degree of adhesiveness was correlated to
the plasma levels of LDL cholesterol ($R=0.7$, $n=33$;
$p<0.0001$; Figure 10).

In an open-label study, 3 hypercholesterolemic
individuals were treated with oral L-arginine
10 supplementation for 2 weeks. Arginine treatment resulted
in a 38% decrease in monocyte adhesiveness.

To confirm this effect of L-arginine treatment and to
control for any experimental bias, a double-blinded,
placebo-controlled, randomized study was performed. Ten
15 hypercholesterolemic subjects were randomized (1:2) to
placebo or L-arginine treatment; 6 normocholesterolemic
individuals were studied in parallel to control for
variation over time in the binding assay. At baseline,
the adhesion of monocytes from both hypercholesterolemic
20 groups was increased in comparison to the
normocholesterolemic individuals ($p<0.001$). After 2 weeks
of L-arginine administration, there was an absolute
reduction of 53% in monocyte binding ($n=7$, $p<0.005$,
baseline vs 2 weeks) (Figure 11). By contrast, there was
25 no significant change in the adhesiveness of monocytes
isolated from hypercholesterolemic individuals treated
with placebo. Two weeks after discontinuation of the L-
arginine treatment, the adhesiveness of the monocytes
isolated from hypercholesterolemic individuals had
30 significantly increased compared to the
normocholesterolemic individuals (34 ± 9% increase in bound
cells/hpf; $p<0.05$), and was also significantly increased
in comparison to the binding obtained after 2 weeks of L-
arginine therapy (an increase of 30 ± 9%, $p<0.05$). The
35 adhesiveness of monocytes from placebo-treated

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hypercholesterolemic individuals did not change
significantly during the washout period.

In some studies monocytes were exposed to sodium
nitroprusside or vehicle control for 30 minutes *in vitro*.
5 Pre-incubation of the cells from hypercholesterolemic
individuals with the NO donor sodium nitroprusside (10^{-5}
M) markedly reduced binding ($164\pm9\%$ vs $98\pm7\%$ vehicle vs
sodium nitroprusside; n=7, p<0.0005; values expressed as a
percent of the normocholesterolemic control exposed to
10 vehicle; Figure 12).

To conclude, the salient findings of this
investigation are that: 1) Hypercholesterolemia enhances
the adhesiveness of monocytes for endothelial cells, 2)
oral arginine supplementation reverses the increase in
15 adhesiveness of monocytes from hypercholesterolemic
individuals, and 3) the effect of oral arginine is
mimicked *in vitro* by exposure of the monocytes from
hypercholesterolemic individuals to sodium nitroprusside,
an NO donor.
20

Example 16. Platelet Hyperaggregability in
Hypercholesterolemic Humans: Reversal by Oral L-Arginine
In this study we tested the hypothesis that chronic
L-arginine supplementation would inhibit platelet
25 reactivity in hypercholesterolemic humans. Venous blood
was collected from normal (NC; n= 11) and
hypercholesterolemic (HC; n=22) volunteers for isolation
of platelet-rich plasma and aggregometry. Half the HC
group received L-arginine (7 g/d) for 2 weeks;
30 aggregometry was performed using collagen (5 mg/ml) before
and after two weeks of treatment.

Results: HC platelets were hyperaggregable. After
two weeks of L-arginine, the aggregability of HC platelets
was reduced (Figure 13). These studies are consistent
35 with our previous observations in animals that oral
administration of L-arginine inhibits platelet reactivity.

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Example 17.   **Intravenous Administration of L-Arginine Improves Endothelium-dependent Vasodilation in Hypercholesterolemic Humans:**

Hyperlipoproteinemia impairs endothelium-dependent
5   vasodilation, even before the development of
atherosclerosis. We hypothesized that administration of
L-arginine may increase synthesis of NO and thereby
improve endothelium-dependent vasodilation in
hypercholesterolemia. Indeed, our earlier studies
10   conducted in cholesterol-fed rabbits support this notion.
The following data demonstrates that L-arginine augments
endothelium-dependent vasodilation in forearm resistance
vessels of hypercholesterolemic humans.

The control subject population in this study included
15   11 normal volunteers comprising (10 males and 1 female).
Their ages ranged from 31 to 49 and averaged 39 ± 2 yr.
There were 14 patients with hypercholesterolemia.
Hypercholesterolemia was defined as a serum LDL
cholesterol level greater than the 75th percentile
20   adjusted for age and sex. These individuals included 11
males and 3 females whose ages ranged from 22 to 48 and
averaged 38 ± 2 years.

Under local anesthesia and sterile conditions, a
polyethylene catheter was inserted into a brachial artery
25   of each subject for determination of blood pressure and
for infusion of drugs. A separate polyethylene catheter
was inserted into the antecubital vein for infusion of L-
arginine. Bilateral forearm blood flow was determined by
venous occlusion strain gauge plethysmography, using
30   calibrated mercury-in-silastic strain gauges, and
expressed as ml/100 ml tissue per min.

To assess NO-dependent vasodilation, methacholine
chloride (which induces the endothelium to release NO) was
administered via the brachial artery. Forearm blood flow
35   was measured during infusion of methacholine chloride at
concentrations of 0.3, 3, and 10 μg/min each for 3 min.

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After completion of the methacholine chloride
infusions, all normal subjects and 10 individuals with
hypercholesterolemia were given L-arginine intravenously
over 30 minutes and then the methacholine infusions were
5   repeated. D-arginine, the enantiomer of L-arginine, is
not a precursor of NO. Thus, to ensure that any observed
effects of L-arginine were due to its contribution to the
synthesis of NO and not just secondary to its
physiochemical properties, five individuals with
10 hypercholesterolemia received D-arginine intravenously.

Results. Baseline blood pressure, heart rate, and
forearm blood flow did not differ between normal and
hypercholesterolemic subjects. Intraarterial infusion of
methacholine chloride caused a dose-dependent increase in
15 forearm blood flow. In the hypercholesterolemic subjects,
however, cholinergic vasodilation was less than that of
normal subjects ($p < 0.05$). The maximal forearm blood flow
response to methacholine in normal subjects is 19.0 ± 1.9
ml/100 ml of tissue per min, and in hypercholesterolemic
20 subjects, it was 13.7 ± 1.7 ml/100 ml of tissue per min
($p < 0.05$).

In the normal subjects, L-arginine did not potentiate
the vasodilation that occurred during the administration
of methacholine chloride. In the hypercholesterolemic
25 subjects, however, the L-arginine infusion augmented the
vasodilation to methacholine chloride by 25% ($p < 0.05$).
There were no complications or side-effects of the L-
arginine infusions.

The important findings in this study are: (a)
30 endothelium-dependent vasodilation (due to the release of
NO) is reduced in forearm resistance vessels of
hypercholesterolemic humans; and (b) intravenous
administration of L-arginine improves endothelium-
dependent vasodilation in these individuals. NO not only
35 causes vasodilation, but it also inhibits platelet

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aggregation and suppresses monocyte adhesion in
hypercholesterolemic humans..

Example 18. Administration of Intravenous L-Arginine
5 Improves Coronary Endothelial Function in Cardiac
Transplant Recipients

        A reduction in coronary NO-dependent vasodilation
occurs in cardiac transplant recipients and may represent
an early marker for the development of graft
10 atherosclerosis. Reduced NO-dependent vasodilation in
response to acetylcholine is an indicator of endothelial
dysfunction and has been attributed to reduced synthesis
or accelerated degradation of endothelium-derived nitric
oxide. We hypothesized that endothelial dysfunction of
15 epicardial coronary arteries at an early stage of coronary
allograft atherosclerosis might be reversed by L-arginine.
The present study tested the hypothesis that
administration of L-arginine, the precursor of
endothelium-derived NO, improves endothelial vasodilator
20 function of coronary conduit and resistance vessels.

        Cardiac transplant recipients scheduled for elective
annual coronary angiography at Stanford University
hospital were screened for possible participation in the
study. The study protocol was approved by the Stanford
25 University Committee on Human Subjects in Medical
Research. All patients gave written informed consent.
Eighteen patients who had cardiac transplantation 1 to 13
years previously were studied.

        Vasoactive medications were discontinued at least 12
30 hours before the study. After diagnostic angiography
revealed no visually apparent coronary stenosis, a guiding
catheter was used to cannulate the left main coronary
artery. An infusion catheter was then advanced over a
Doppler flow velocity guide wire into a nonbranching
35 segment of the coronary artery for infusion of
acetylcholine (which stimulates the endothelium to release

51

NO). After baseline angiography was performed, increasing concentrations of acetylcholine were serially infused over 3 minutes. Infusion of acetylcholine continued until the maximum dose (10^{-4} mol/L) was reached or until total
5   coronary occlusion occurred. Then an intravenous infusion of L-arginine (30g over 15 minutes) was performed. Thereafter, the intracoronary infusion of acetylcholine was repeated. Coronary angiography and Doppler flow velocity recording was performed at the end of the L-
10 arginine infusion and after the infusion of each concentration of acetylcholine.

Results. In epicardial coronary arteries of these transplant recipients, acetylcholine caused vasoconstriction. Epicardial coronary vasoconstriction
15 caused by acetylcholine was attenuated by infusion of L-arginine (10^{-4} mol/L, -6.8% versus -2.8%; $p<.01$). In coronary resistance vessels, acetylcholine induced vasodilation, reflected by increases in blood flow. The increase in coronary blood flow was significantly enhanced
20 with L-arginine ($p<.002$; Figure 14). There were no complications or side-effects of the L-arginine infusion.

The coronary vasculature of cardiac transplant recipients exhibits a generalized reduction of NO-dependent vasodilation. L-arginine improves endothelial-
25 derived NO dependent vasodilation of both coronary microvasculature and epicardial coronary arteries.

It is evident from the above results, that by enhancing the nitric oxide levels, by means of nitric oxide precursor compounds or other compounds in the nitric
30 oxide pathway, substantial benefits will ensue to patients with vascular degenerative diseases. This treatment will restore normal vascular tone (preventing excessive vasoconstriction and elevation of blood pressure; and will improve blood flow to the heart, brain, and other critical
35 tissues thereby enhancing exercise tolerance and relieving symptoms such as angina or cerebral ischemia); and will

52

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diminish the formation of atherosclerotic plaque and
restenosis (by inhibiting adhesion of monocytes and
platelets, and by reducing the proliferation of vascular
smooth muscle cells). Benefits may also ensue to normal
5 individuals, ecause NO is critically involved in
exercise-mediated vasodilation, an enhancement of NO
synthesis could improve blood flow and exercise capacity
even in normal individuals.

By virtue of administering to the host, based on a
10 predetermined regimen, or providing in the host a supply
of a component in the synthetic pathway for production of
nitric oxide, so as to maintain a mildly elevated level of
nitric oxide in the host, particularly at the site to be
treated, the incidence of plaque formation can be
15 substantially diminished. This can be achieved in a
variety of ways: by oral administration in accordance with
a predetermined regimen of various compounds associated
with nitric oxide formation, e.g. L-arginine and/or L-
lysine; by administration at the site, in a predetermined
20 regimen of compounds which can produce nitric oxide,
either directly or as a result of physiologic action of
endogenous compounds, e.g. enzymes; by employing
combinations of compounds, which by their action result in
the production of nitric oxide; or the like. These
25 individual administrations, can be done independently or
in conjunction with a regimen of other compounds
associated with the production of nitric oxide.

Alternatively, one may use genetic engineering to
introduce a gene associated with a component in the
30 synthetic pathway for production of nitric oxide, e.g.
nitric oxide synthase, where the enhanced production of
such compounds will have the effect of driving the
equilibrium to an enhanced production of nitric oxide.
Thus, the subject invention provides a plurality of
35 pathways to enhance the synthesis or action of nitric
oxide, or reduce the degradation of nitric oxide, thereby

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increasing the effect of endogenous nitric oxide to
prevent the formation of vascular lesions and to inhibit
restenosis.

5   All publications and patent applications cited in
this specification are herein incorporated by reference as
if each individual publication or patent application were
specifically and individually indicated to be incorporated
by reference.

     Although the foregoing invention has been described
10   in some detail by way of illustration and example for
purposes of clarity of understanding, it will be readily
apparent to those of ordinary skill in the art in light of
the teachings of this invention that certain changes and
modifications may be made thereto without departing from
15   the spirit or scope of the appended claims.

54

WHAT IS CLAIMED IS:

1.   A composition for use in a method of improving function and structure of the vascular system of a human host, said method comprising:

5   administering orally to said host in accordance with a predetermined regimen a prophylactic dose of a source of L-lysine as other than a natural food source and as a dietary supplement to enhance the level of endogenous NO in the vascular system to improve vascular function.

10

2.   A composition according to Claim 1, wherein said dose comprises at least 10% by weight of L-lysine and at least 50% total weight of amino acids L-lysine and L-arginine, polypeptides comprising at least about 40 mol%

15 of said amino acids, or physiologically acceptable salt thereof.

3.   A composition according to Claim 2, wherein said polypeptide is an oligopeptide of L-arginine and L-lysine.

20

4.   A composition according to Claim 2, wherein said dose comprises L-arginine.

5.   A composition according to Claim 4, wherein said

25 dose is administered in a daily amount in the range of 0.5 to 25g per day.

6.   A composition according to Claim 1, wherein said dose comprises at least one of calcium, an amino acid

30 absorption enhancing compound, a cofactor for NO synthase activity, or an antioxidant in an amount sufficient to enhance the prophylactic effect of said L-lysine.

7.   A composition according to any of Claims 1-6,

35 wherein said dose is administered as a tablet, capsule, or powder.

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8.   A composition according to any of Claims 1-6,
wherein said dosage is administered as a prepared solid
food, nutritional supplement or liquid.

5

9.   A physiologically acceptable formulation
comprising L-lysine and L-arginine of which L-lysine is
at least 10 weight %, or their physiologically acceptable
salts in from about 0.5 to 25g, and at least one of
10   calcium, folate, B_6,   or B_{12}, in sufficient amount to
enhance the effect of said L-arginine, L-lysine or their
physiologically acceptable salts on enhancing the amount
of NO in a human host.

15   10.   A composition for use in a method for inhibiting
vascular smooth muscle cell proliferation at a site of
injury in the vascular system, said method comprising:
        administering at said site an effective amount of at
least one of L-arginine, L-lysine or its physiologically
20   acceptable salt and not less than 10 weight % L-lysine or
its physiologically acceptable salt to enhance NO
production;
        whereby vascular smooth muscle cell proliferation is
inhibited.
25

11.   A composition according to Claim 10, wherein
said injury is as a result of angioplasty.

12.   A composition for use in a method for reducing
30   the probability of restenosis resulting from injury caused
by angioplasty or atherectomy, said method comprising:
        introducing intramurally proximal to the site of
said injury a nitric oxide precursor at a time and in an
amount to increase the amount of nitric oxide formation of
35   the cells proximal to said injury,
        whereby a reduced incidence of restenosis occurs.

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13.   A composition according to Claim 12, wherein
said nitric oxide precursor is at least one of L-arginine,
L-lysine or an oligopeptide comprising at least one of L-
arginine and L-lysine.

5

14.   A method according to Claim 12, wherein said
time is at the time of said injury and not later than four
weeks thereafter.

12.
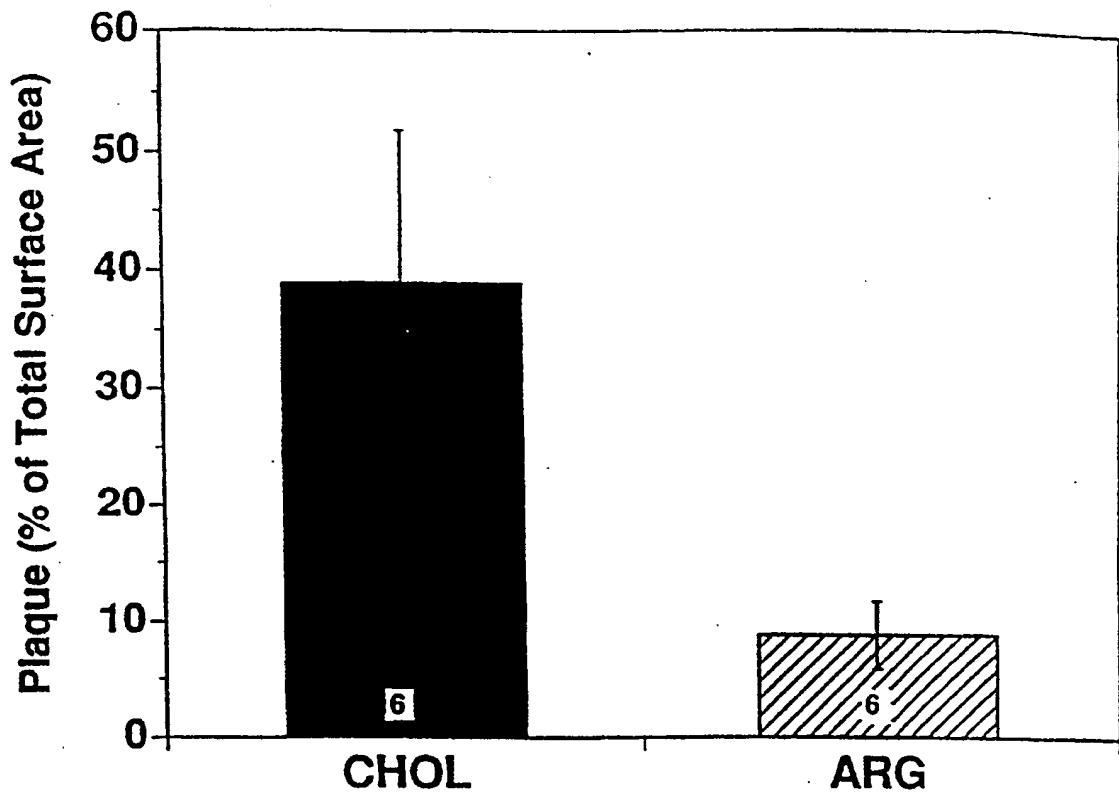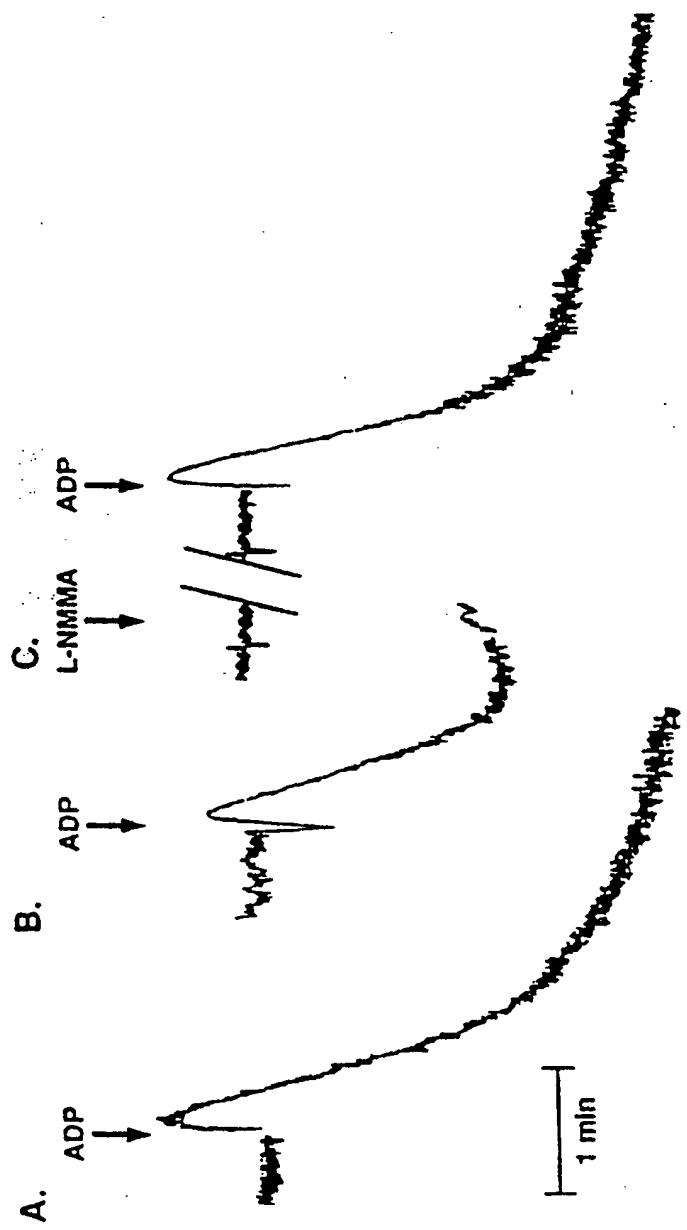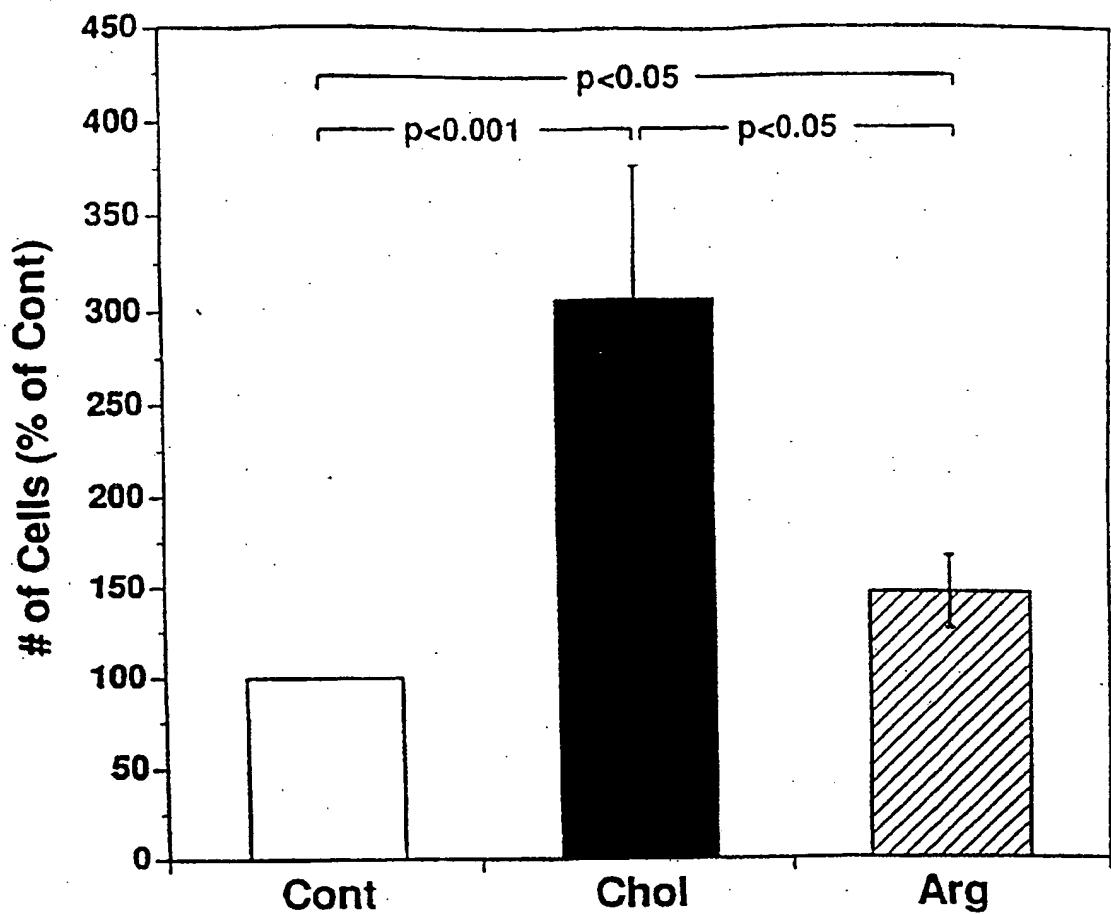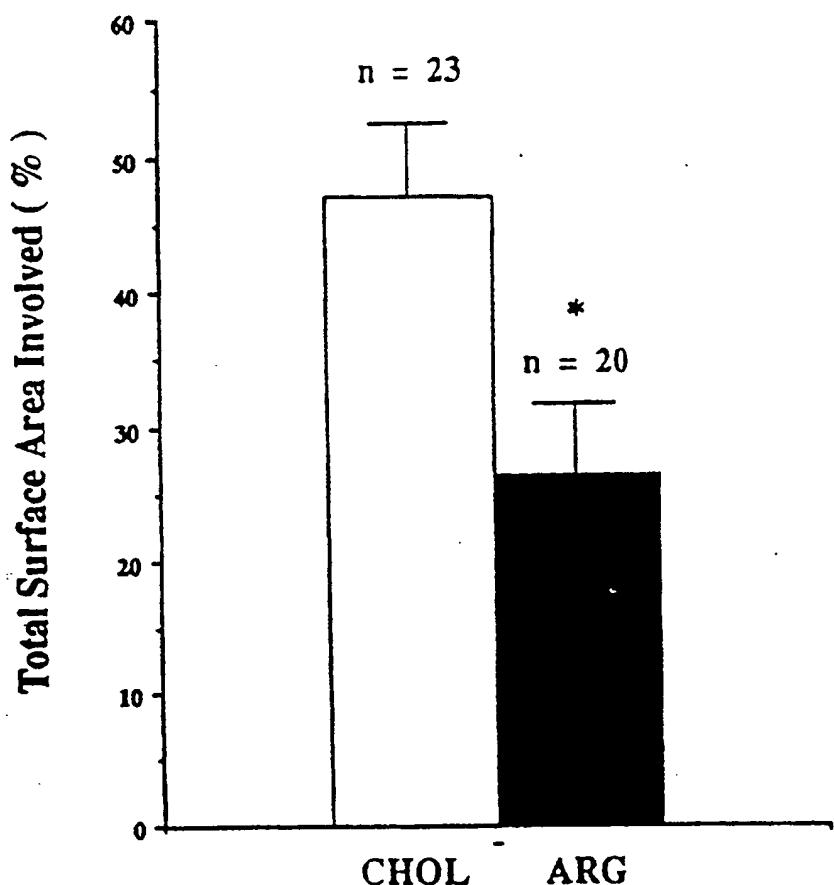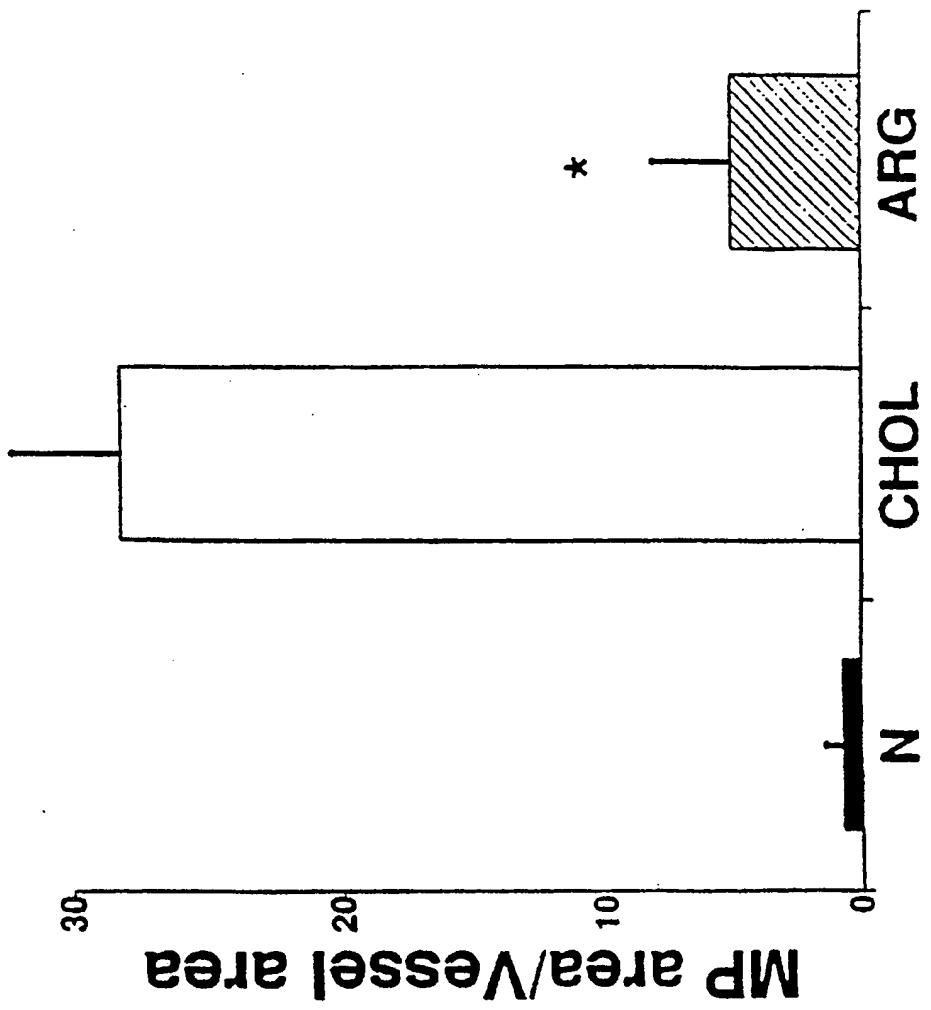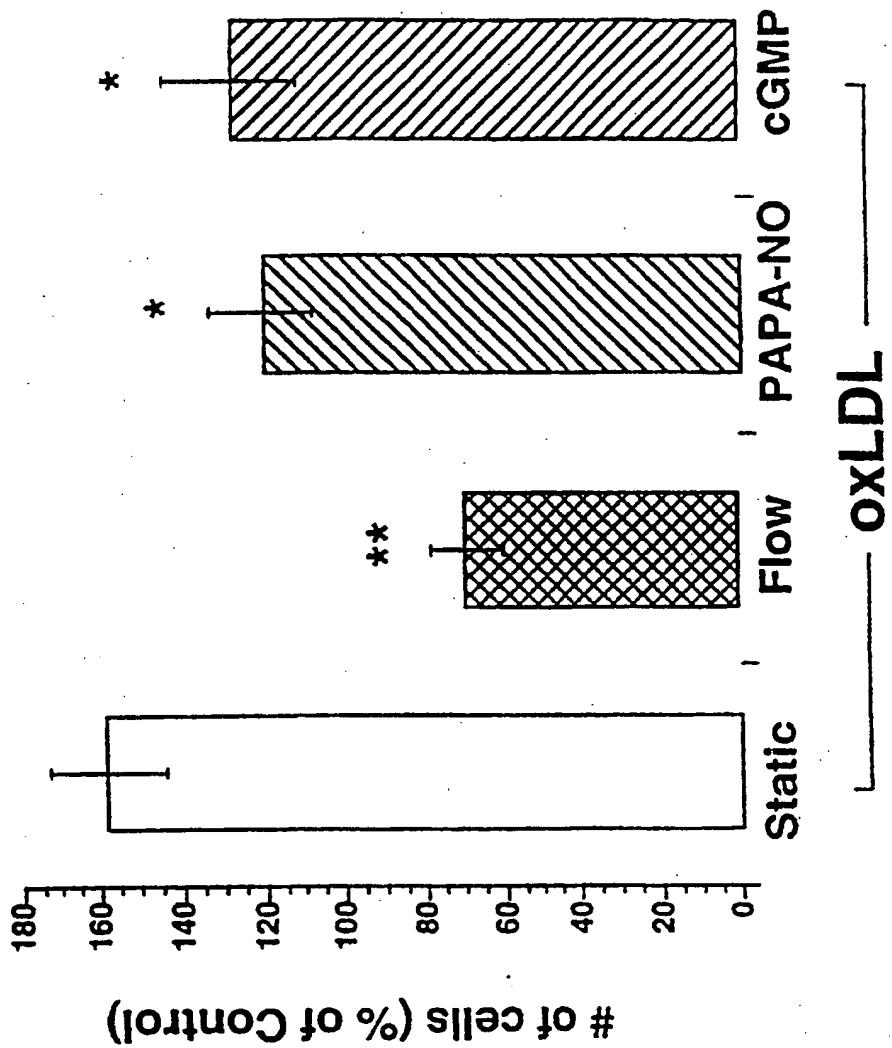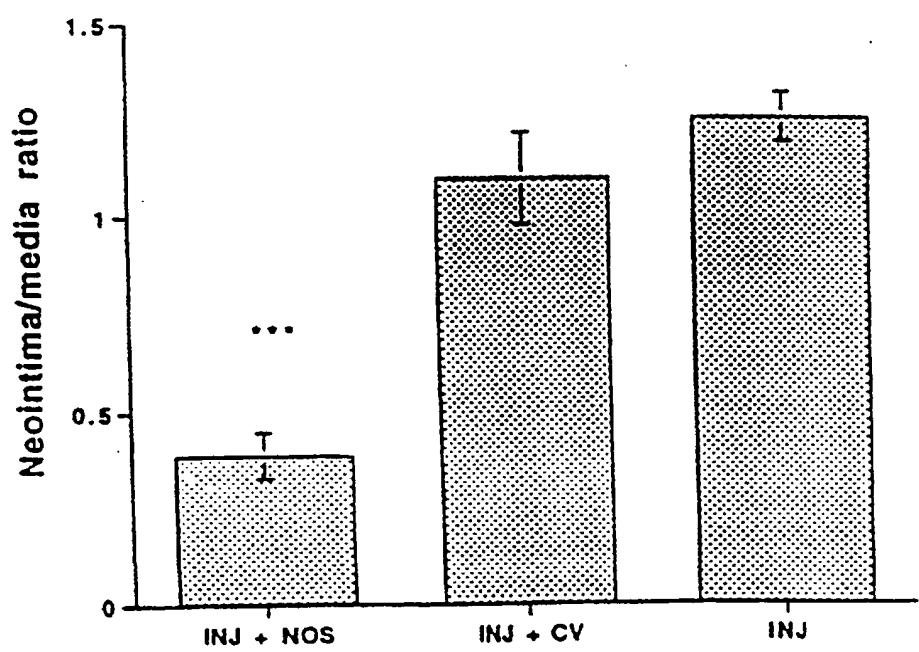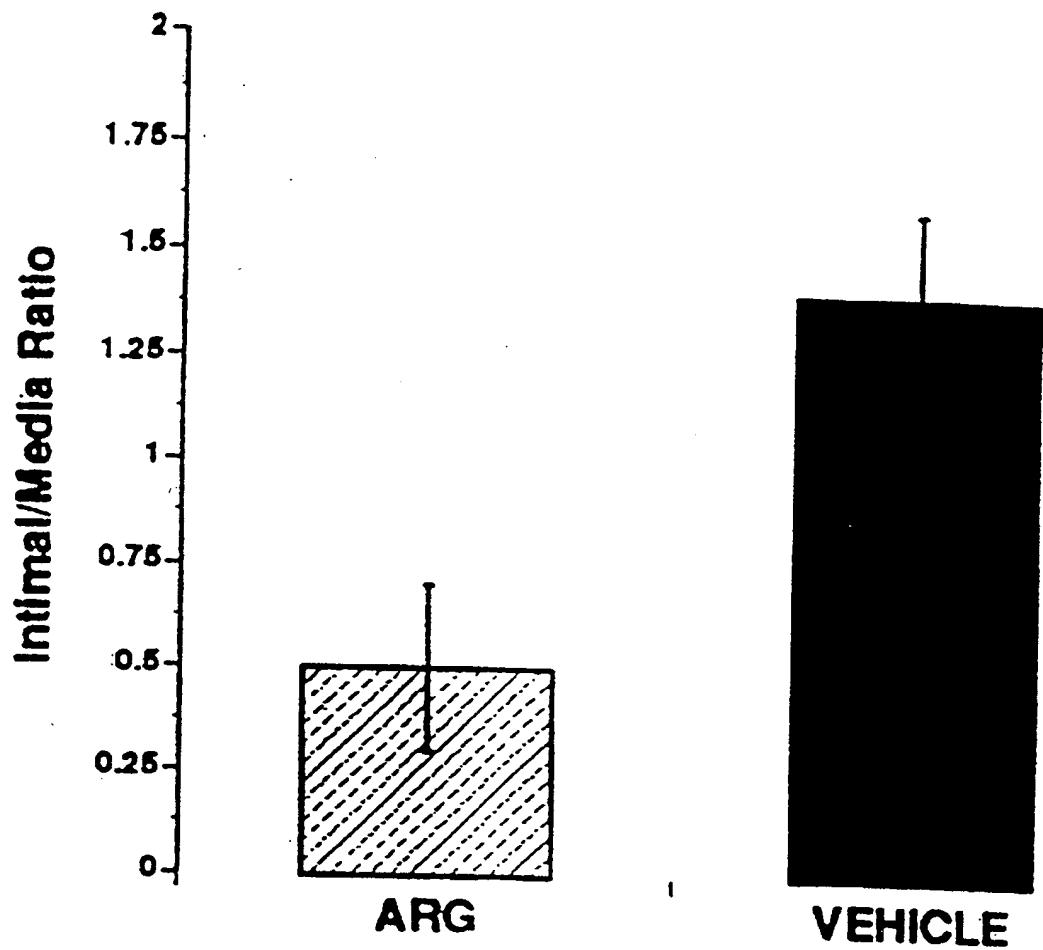
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FIG 1

FIG 2

FIG 3

4/15

FIG 4

FIG 5

FIG 6

FIG 7

FIG 8

FIG 9

FIG 10

FIG 11

FIG12

FIG 13

■ NC ▩ HC$_A$

Aggregation (%)

baseline: 54.5, 70.9 (p<0.01)

2 weeks: 51.3, 65.6 (p<0.01)

4 weeks: 55.1, 52.6

Coronary Blood Flow
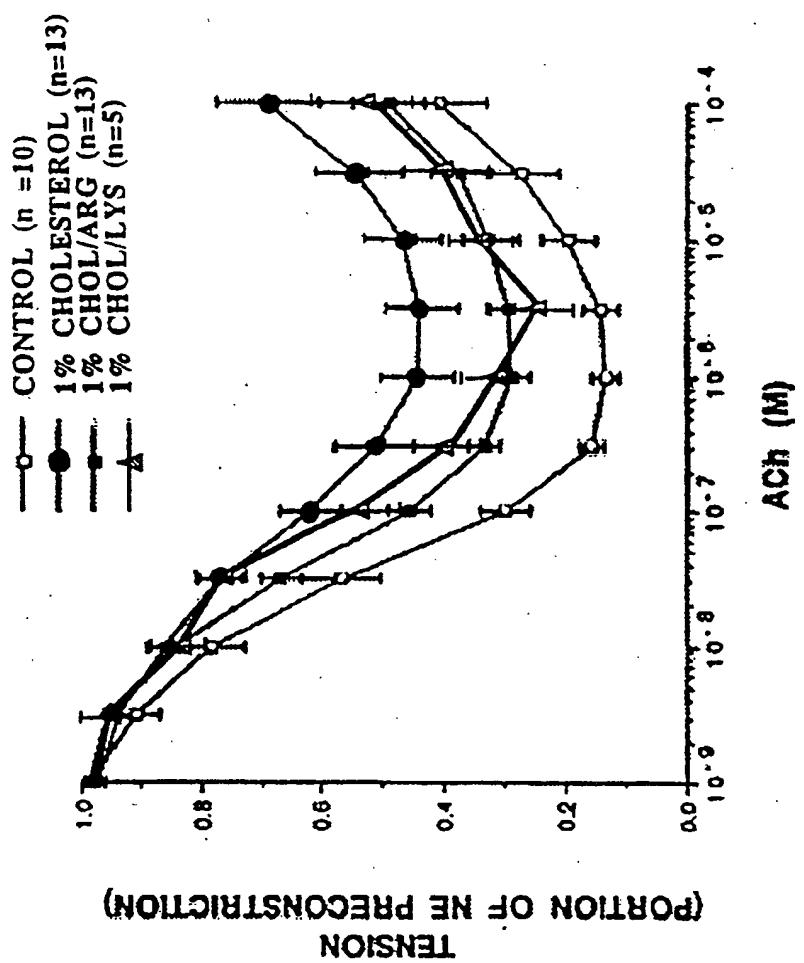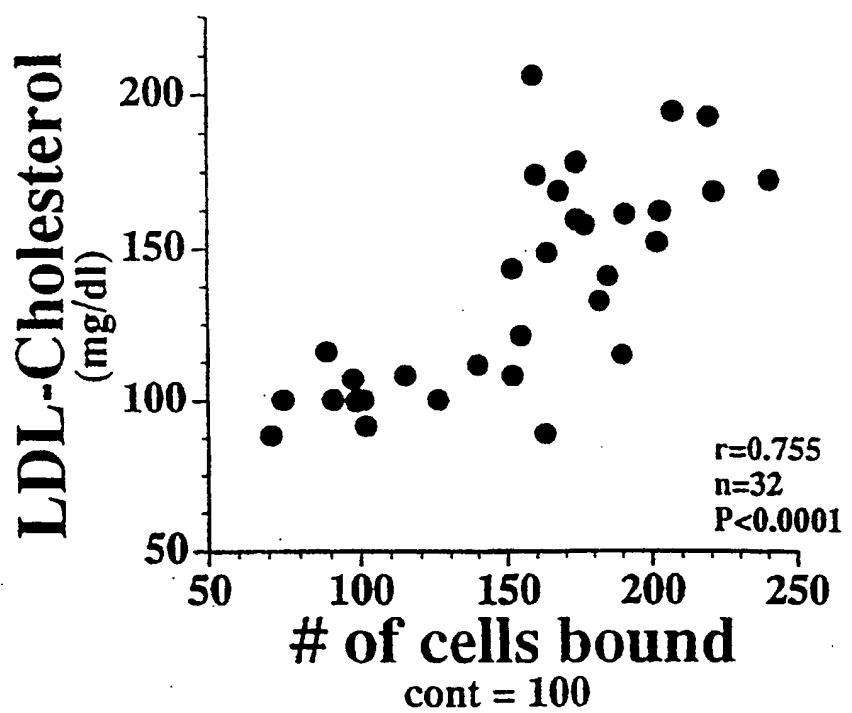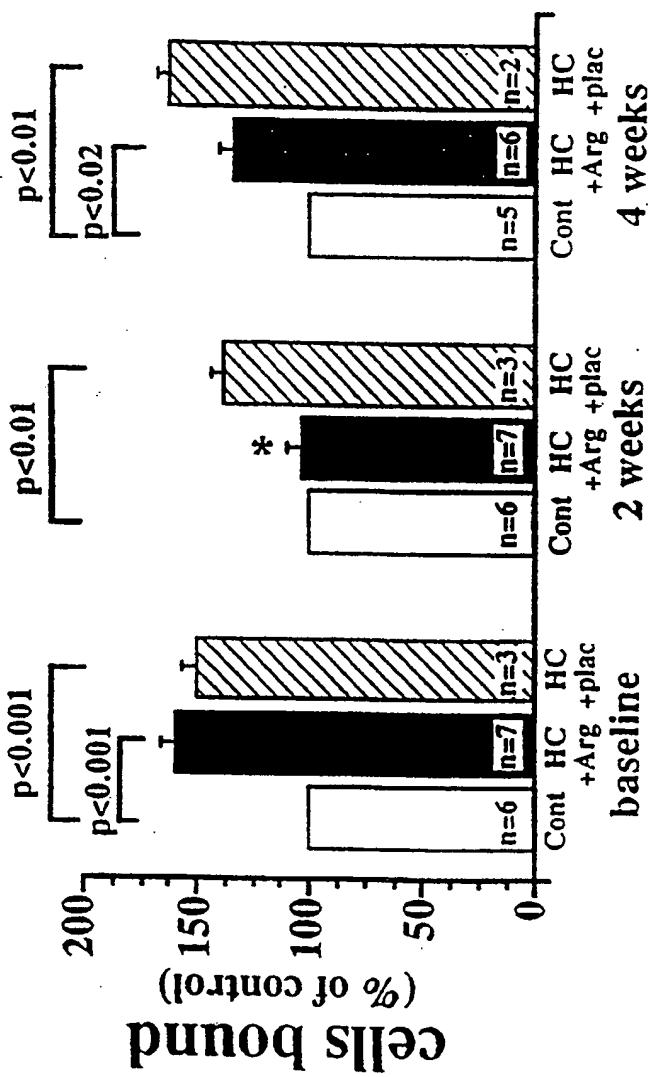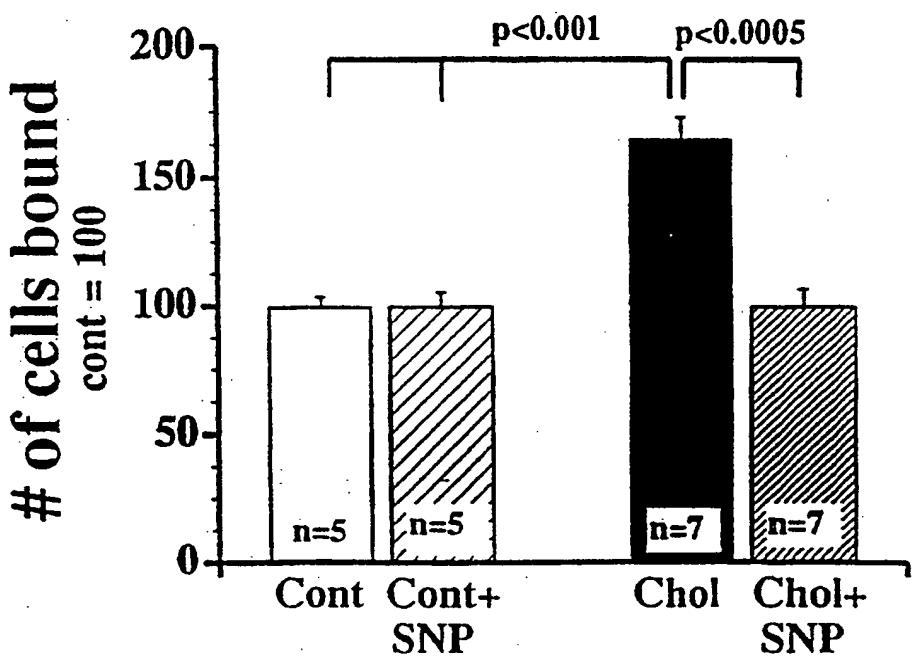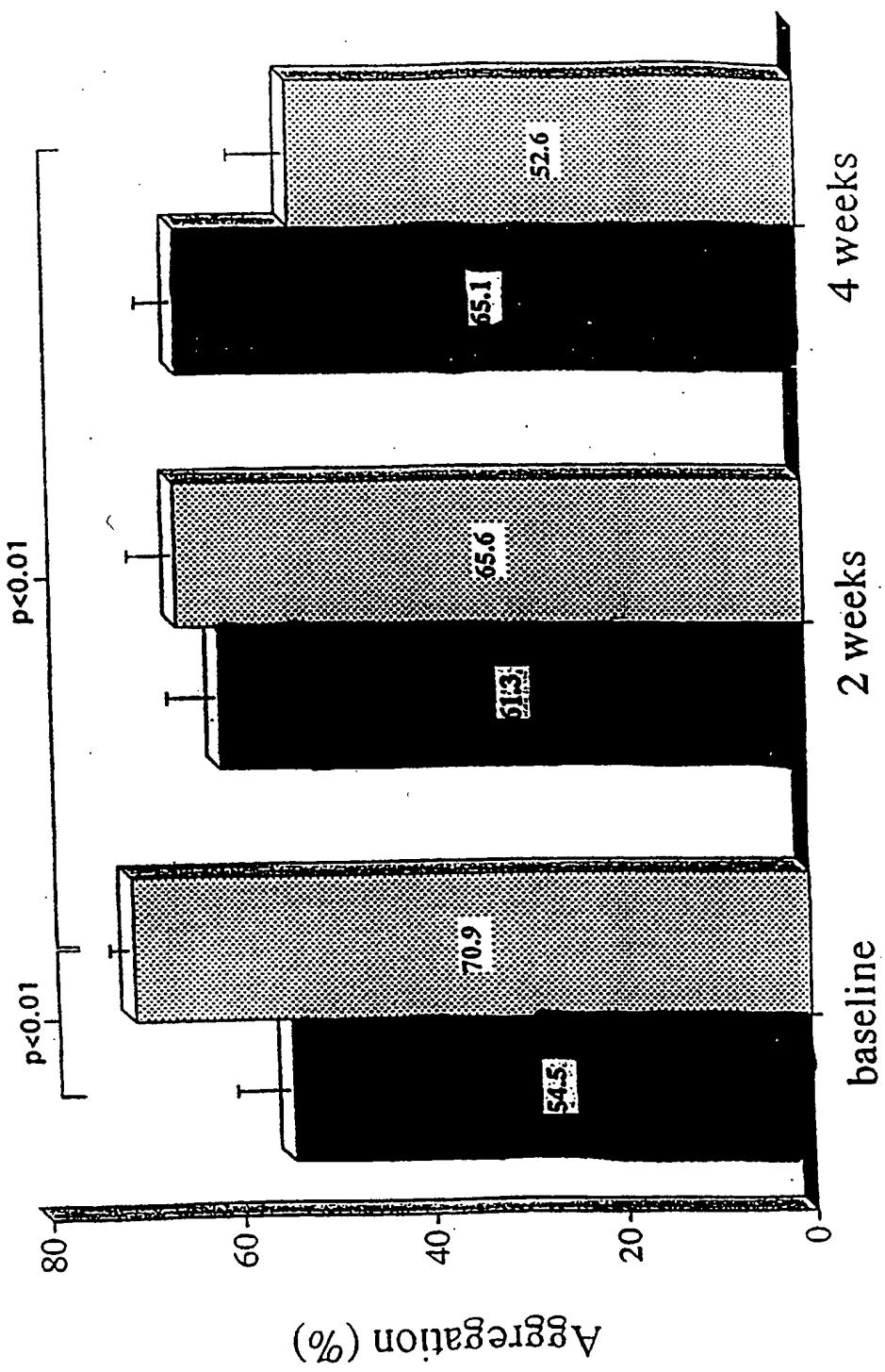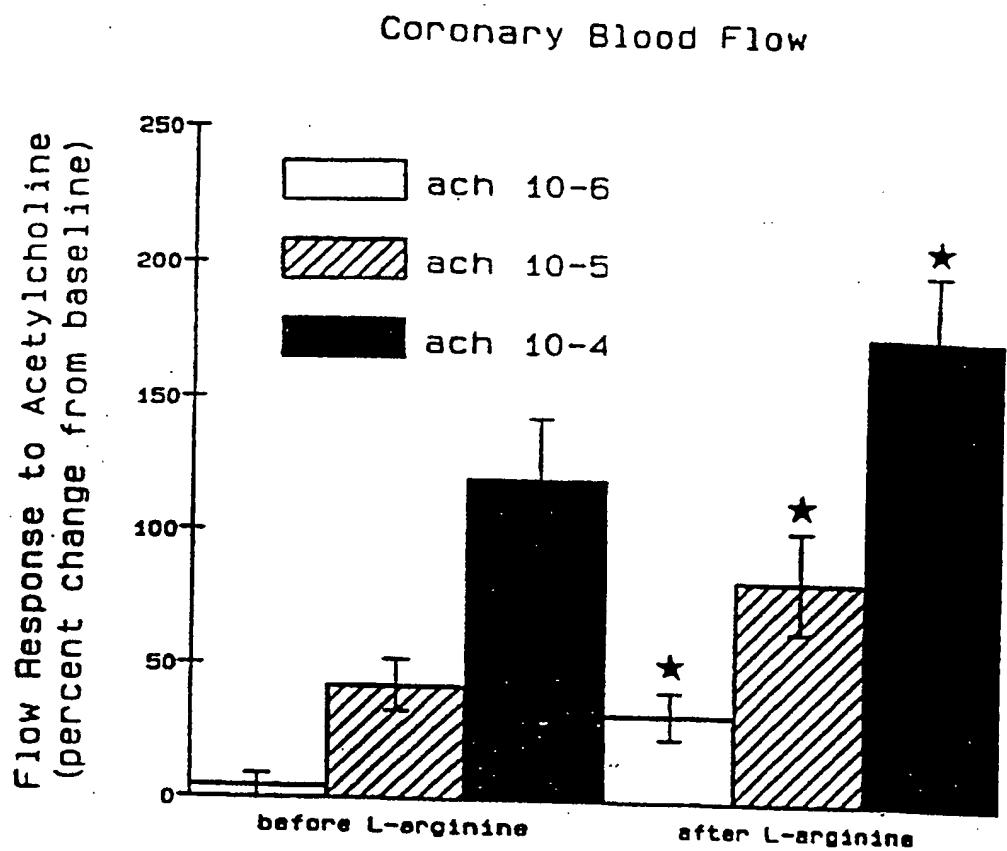
**FIG 14**
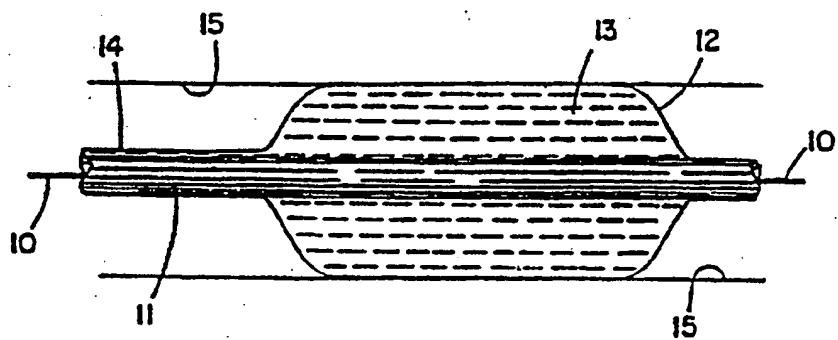
FIG. 15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/17241

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A23L 1/305; A61K 31/195, 38/02

US CL :426/648, 656; 514/20, 310, 557, 564, 565

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 426/648, 656, 657; 514/20, 310, 557, 564, 565; 530/358; 930/290

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

search terms: arginine, lysine, polyarginine, polylysine, vascular, vasodilate, atherosclerosis, thrombosis, restenosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,428,070 A (COOKE ET AL) 27 June 1995(27/06/95), see entire document.	10-14
X	EP 0,546,796 A (AJINOMOTO CO., INC.) 16 June 1993 (16/06/93), see entire document.	10-14
X	US 4,920,098 A (COTTER ET AL) 24 April 1990 (24/04/90), column 4, lines 2-18, column 7, lines 1-3, Examples 1-3.	1, 6-8, 10-14
X	US 5,032,608 (DUDRICK) 16 July 1991 (16/07/91), column 13, lines 48-60.	1, 6, 8, 10-14
X, E	US 5,576,287 A (ZALOGA ET AL) 19 November 1996 (19/11/96), column 5, line 38 - column 6, line 3.	1, 6, 8, 10-14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 JANUARY 1997	21 JAN 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized Officer JEFFREY E. RUSSEL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,278,189 A (RATH ET AL) 11 January 1994 (11/01/94), column 2, lines 14-34, column 4, lines 43-49, Tables 1-2, claims 1-15.	1, 2, 4-8, 10-14
X	US 4,029,773 A (BEIGLER ET AL) 14 June 1977 (14/06/77), abstract, column 6, lines 27-63.	1, 2, 4-6, 8-14
X	US 4,847,240 A (RYSER ET AL) 11 July 1989 (11/07/89), claims 4-6.	1-3, 8, 12-14